Anticancer immunomodulatory activity of Melaleuca

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Published

2019-12-20

Thesis Type

Thesis (Masters)

School

School of Medical Science

DOI

10.25904/1912/2125

Anticancer immunomodulatory activity of Melaleuca

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A thesis submitted in fulfilment of the requirements for the degree of
Master of Medical Research Candidate
in
Biochemistry and Cell Biology

August 2019

ABSTRACT

The immune system plays a critical role in disease prevention via collaboration between the adaptive and innate immune responses. Due to a variety of immune cell subpopulations with different functions including opposing roles, the tumour microenvironment provides opportunities as a therapeutic target for anticancer drug development. Recently the essential oils and their extracts from plants have been gaining attention in cancer research, due to their antioxidant and anti-inflammatory activities making them good candidates as sources of novel anticancer compounds. The essential oil of *Melaleuca Alternifolia* and its derived products have been previously reported to cause tumour regression, accompanied by an accumulation of neutrophils at treatment sites in various studies. Therefore, the present study was aimed at investigating the immunological responses induced by intratumoural injection of *Melaleuca Alternifolia* Concentrate (MAC) into subcutaneous tumours of the female transgenic FVB/N c-neu spontaneous murine model of breast cancer, particularly focusing on the role of neutrophils.

Following emerging evidence demonstrating the anticancer activity of neutrophils under different stimulants, the present study investigated the immune responses induced by directly injecting MAC into solid tumours of transgenic FVB/N c-neu mice. 4 % v/v MAC was injected directly into the subcutaneous tumours of the female mice and significantly slowed growth of tumours with no observable side effects. The results showed increased levels of neutrophils purified from the treated tumours as compared to vehicle control and untreated control tumours. The results were consistent with previous studies which reported an increased influx of neutrophils into the tumours following MAC injections.

The present study further characterised the neutrophils isolated from treated tumours, and the results showed a phenotypic diversity of neutrophils isolated from tumours mostly containing a hypersegmented nucleus constituting the bulk (62.76 %) of

the cytotoxic high-density neutrophils. Emerging evidence has indicated the phenotypic diversity and heterogeneity of neutrophils in cancer. Furthermore, this study evaluated the cytotoxicity of the neutrophils isolated from treated tumours, *ex vivo*. Neutrophils isolated from tumours treated with 4 % v/v MAC showed cytotoxicity towards NeuTL murine breast cancer cells *in vitro* and effective killing by neutrophils at effector-to-target ratios from10:1 down to 1.25:1 ($p<0.001$).

In summary, the present study found that 4 % v/v MAC impairs the growth of tumours and with no observable toxicity in mice. Also, neutrophils activated in response to treatment of FVB/N tumours with MAC are cytotoxic for NeuTL murine breast cancer cells and are characterised by a hypersegmented nuclear morphology. At present, literature on specific anti-tumorigenic roles played by TANs in the tumour microenvironment is limited. Thus, data from this study contributes towards identification of specific roles and mechanisms underlying the chemoattraction of TANs into the tumour microenvironment and more importantly in response to MAC intervention.

Statement of Originality

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

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Chandi Tabeth Magawa

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ACKNOWELEDEMENTS

With great appreciation and love, I would like to express my heartfelt and sincere gratitude to all the people who helped and supported me throughout this study in including the following:

My principal supervisor Associate Professor Stephen Ralph, for his invaluable guidance and support throughout this research and 98 Alive Pvt Ltd for supplying me with MAC oil and financial assistance;

The animal facility staff, in particular Guy Beford for his profound assistance with my animal work, Hayley Duncan and Dr Allanah McMath for their assistance with breeding and caring for the animals and Janet Currie for her assistance with training for animal care and handling;

The School of Medical Science staff, G12 laboratory and technical staff, in particular Dr Ruth Lambrechts and Dr Jelena Vider for their assistance and training with flow cytometry work;

The past and present Ralph laboratory members and fellow students, in particular Reem ALHulais for her general assistance with laboratory work;

My long list of friends for their exceptional moral support;

Last but not least, to my family in particular Humphrey, Keisha and Michael for their patience, understanding, motivation and unending moral support.

List of abbreviations

4T-1	Murine breast cancer cell line
AE17	Human mesothelioma cell line
AK	Actinic keratosis
Arg1	Arginase 1
ANOVA	Analysis of variance
ATCC	American Type Culture collection
CCL	Chemokine Ligand
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen 4
CXCL	Chemokine (C-X-C motif) ligand
DAPI	4'-6-Diamindo-2-phenylindole
DC	Dendritic cell
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraaccetic acid
ELISA	Enzyme-linked immunosorbent assay
Foxp3	Forkhead box p3
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte monocyte-stimulating factor
HER2	Human epidermal growth factor receptor 2
ICAM	Intracellular adhesion molecule
IFN	Interferon
IL	Interleukin
i.t	Intratumoural
LNCaP	Human prostate cancer
LPS	Lipopolysaccharide
M14	Human melanoma
mAB	Monoclonal antibody
MAC	*Melaleuca alternifolia* Concentrate
MCF-7	human breast cancer cell line
MDSC	Myeloid-derived suppressor cells
MOLT-4	Human lymphoblastic leukaemia T-cell cell line
MPO	Myeloperoxidase
N1	Immunogenic neutrophil
N2	Tolerogenic neutrophil
NET	Neutrophil extracellular trap
NK	Natural killer

NLR	Neutrophil-to-lymphocyte ratio
NO	Nitric oxide
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PD1	Programmed cell death protein 1
PDL1	Programmed cell death ligand 1
ROS	Reactive oxygen species
T4ol	Terpinene-4-ol
TAN	Tumour associated neutrophil
TAM	Tumour associated microphages
TGF-β	Tumour growth factor beta
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TPGS	D-alpha Tocopherol polyethylene glycol 1000 succinate
Treg	Regulatory T cell
TTO	Tea tree oil
TLR	Toll-like receptor
VEGF	Vascular endothelial growth factor

Subtitle: Role of tumour associated neutrophils in cancer therapy using a *Melaleuca Alternifolia* extract

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Chapter 1: Introduction

Background

The tumour microenvironment is characterised by a variety of cells including host stromal cells, secreted factors, extracellular matrix proteins as well as immune cells. Although the immune system has evolved to protect the host from harmful agents, dealing with cancer cells appears to be challenging. Evidence for the role of immune cells within the tumour microenvironment is contradictory, with various immune cells having different functional roles or even playing opposing roles [1]. For instance, in the early stages of malignancy, macrophages have been known to have antitumor functions whereas when the disease progresses, they adopt tumour growth supporting functions [2, 3] . Likewise, previously the role of neutrophils in cancer has been shown to support cancer growth [4]. However, in recent years emerging evidence has arisen showing the heterogeneity of neutrophils in cancers and the varying roles they play in different types or even stages of cancers [5, 6]. Similar to the macrophages that display antagonistic functional activities [7], neutrophils have also been shown to undergo an immune switching process in response to different stimulants within the tumour microenvironment, assuming different phenotypes with opposing functions in different cancers [6, 8]. In some cancers, a high neutrophil count is associated with poorer prognosis such that the ratios of circulating neutrophils to lymphocytes are used to determine the prognosis for the disease [4, 9].

Current anti-cancer treatment therapies have been successful at improving clinical outcomes of patients with cancer and saving lives. However, they have limitations which include; development of multidrug resistance, adverse side effects and continued lower success rates than desired. Therefore, recent studies have been focusing on developing novel anticancer compounds and alternative treatment methods that are both safe and effective. Investigations of anticancer drugs have also been focusing on developing therapeutics derived from natural products such as plants and essentials oils with the expectation that they may provide improved clinical outcomes and reduced costs. Several studies have highlighted the effectiveness of plant-derived therapies and how

some may potentially modulate the immune system and these will be further discussed below.

In recent years, extracts including terpene components have been derived from the oils of the Australian native plant *Melaleuca alternifolia*, commonly known as Tea tree oil (TTO) and have gained interest in medical research due to their antimicrobial and anticancer properties [10-12]. The anticancer activity of *Melaleuca alternifolia* has been established in various studies both from *in vitro* and *in vivo* studies, with reportedly reduced adverse effects towards the healthy cells [13, 14]. In pre-clinical trials using murine tumour models, *Melaleuca Alternifolia* and its main active compound terpinen-4-ol have been reported to cause tumour regression associated with the presence of infiltrating immune cells around the treatment sites [14-16]. Also, the topical application of pure TTO in dilute dimethyl sulphoxide (10 %TTO/DMSO) to subcutaneous melanoma bearing mice was reported to exert significant inhibition of tumour growth and tumour clearance was found to be associated with skin irritation mediated by neutrophils, with the irritation and tumours quickly and completely resolved after cessation of treatment [16].

In yet another study at Griffith University reported by A. Clark (2013, in her PhD thesis), intratumoural injections of *Melaleuca Alternifolia* Concentrate (MAC) into subcutaneous tumours of FVB/N c-neu spontaneous murine breast cancer models was found to cause tumour regression with an observed elevated neutrophil production and tumour infiltration [14]. With mounting evidence to support the diversity of tumour associated neutrophils (TANs) in cancers, the current study was aimed at further investigating the role of these TANs using the transgenic FVB/N spontaneous murine breast cancer model.

Aims and Objectives

The project was aimed at expanding the preliminary findings from previous studies at Griffith University on *Melaleuca Alternifolia* Concentrate (MAC). The purpose of the project was to further investigate the immunomodulatory activity of MAC following

injections intratumourally into subcutaneous tumours of transgenic FVB/N spontaneous murine breast cancer model. The objective was to identify the immune cell subpopulations present in the tumour microenvironment particularly focussed on the types of neutrophils and lymphocytes, followed by nuclear morphological examination of the infiltrating neutrophils. Furthermore, it sought to evaluate the activated tumour TANs *ex vivo*, post treatment for their cytotoxicity activity against the breast cancer cells.

The project was aiming to address the following questions;

1. Does injecting MAC into subcutaneous tumours of murine breast cancers induce an immunological response against the tumour cells?
2. What type of tumour associated neutrophils (TANs) are activated in response to MAC treatment?
3. Do activated tumour infiltrating neutrophils have cytotoxic effects against the tumour cells?

Context of study

The basis of the study was to follow previously used protocols (Clark, Griffith University PhD, 2013), with some adjustments made and addition of new protocols. The aim of the project was to determine the cytotoxic activity of MAC treatment against the murine breast cells *in vitro* using SYTOX Green-based assays. *In vivo* studies sought to investigate the immune response elicited by MAC against tumour cells in the transgenic FVB/N spontaneous murine breast cancer model by examining and identifying changes in various immune cell subpopulations after treatment that appeared within the tumour microenvironment. The project was focused on analysing the infiltrating TANs, by examining their nuclear morphology and their ability to mediate elimination of cancer cells. For the latter approach, the isolated TANs were analysed for their tumour specific killing activity against a breast cancer cell line derived from the strain known as Neu TL

cells when these target cells were grown in culture. The TANs found in the tumours were identified as positive for the surface markers $CD11b^+$, $Ly6G^+$. The study also investigated the expression of the surface receptor, c-MET on the $Ly6G^+CD11b^+$ cells. The latter aspect emerged following a study by Finisguerra et.al 2015, which reported elevated c-MET expression levels on circulating cytotoxic TANs isolated from treatment of melanoma bearing mice and non-small-cell lung tumours when compared to healthy mice, suggesting that MET expression is required for neutrophil chemoattraction and cytotoxicity [17].

It was recognized that the tumour targeting by specific subsets of cells, in particular neutrophils, with their known short half-lives could be a challenging proposal. However, several studies were reported suggesting that murine neutrophils are actually capable of having a longer life span, provided that their environment is free of any activation stimulants. Therefore, one important consideration for this work was that it would be required to avoid any possible purification and preparation based-activation of neutrophils during the course of the analyses.

Significance of study

At present, literature on the specific anti-tumorigenic roles played by TANs in the tumour microenvironment is limited. Hence; this study was aiming to contribute towards identification of specific roles played by TANs isolated from the tumour microenvironment and more importantly in response to MAC intervention. Furthermore, the study was aiming to examine the differential neutrophil activation when using natural based treatments for cancer and how such a strategy could be potentially harnessed for cancer therapies.

With interest growing in the development of novel cancer therapeutics to possess both direct tumour cell killing as well as immune enhancing properties, a greater understanding of the role played by TANs against tumour cells would provide an opportunity for developing improved cancer therapeutics with double-edged sword like characteristics. This project was proposed to extend on the previous studies using *Melaleuca Alternifolia* concentrate (MAC). Furthermore, the study would add to the body of studies aiming to identify potential anticancer therapies which can be combined in conjunction with surgical therapies, with the aim of improving the clinical outcomes of patients with cancer. Injecting chemotherapy drugs with both direct killing effects and immune enhancing properties could be used to reduce the local tumour burden prior to surgery, reducing requirements for extensive surgery of inoperable cancers. More so, immune enhancing cancer treatments could also be used to eradicate residual tumours post-surgery.

Thesis outline

Chapter 2: Literature review
Chapter 3: Materials and Methods
Chapter 4: Results
Chapter 5: Discussion and Conclusion

Chapter 2: Literature review

Topic 1: Cancer overview

Interference in the normal balance of the cell cycle due to multiple factors such as carcinogens, radiation, bacteria and viruses; leads to uncontrolled cell division resulting in the development of cancer [18-20]. The stages and processes which lead to the transformation of normal human cells into malignant cells have been extensively investigated over the years, and evidence shows that most types of cancer share similar biochemical, molecular and cellular traits as they evolve [21, 22]. In essence, different types of cancer acquire functional capabilities at different stages of the multistep tumorigenesis, which are critical for their survival, proliferation and dissemination. The hallmarks of cancer have been extensively reviewed and first proposed as the ability to sustain proliferative signalling, enable replicative immortality, sustain angiogenesis, reprogram metabolism energy, programmed cell death and the ability to activate invasion and metastasis [22]. A decade later, as new evidence emerged, the ability to evade growth suppressors and immune destruction were also added to the earlier proposed list [21].

Although anticancer therapeutic approaches have evolved over the years, deaths from cancers are still on the rise. According to the World Health Organization (WHO) in 2018 alone, an estimated 9.6 million deaths worldwide resulted from cancer, placing cancer in second place for causing the most deaths globally, with cardiovascular disease leading the list [23]. Furthermore, the Australian Institute of Health and Welfare 2019 data predicted an increase in the incidence of new cancers in Australia in 2019 to reach 483 per 100 000 people, with breast cancer and prostate cancer as the most commonly diagnosed forms of cancer [24]. However, deaths related to cancer are expected to decline to 159 per 100 000 people, owing to the current improvements in cancer research leading to development of treatments [24].

At present, several methods and strategies are being used in single therapy or in combination therapy, in the fight against cancer including chemotherapy, radiation, surgery, targeted therapy, stem cell transplant, precision medicine and immunotherapy.

Most current chemotherapeutic regimens usually use highly cytotoxic compounds to target cancer cells and although highly effective, their successful results are often accompanied by detrimental side effects on the normal cells due to their non-discriminatory nature [25]. Furthermore, drug sensitivity differences among tumours, long treatment regimens and high rates of incomplete tumour clearance are still some of the limiting factors with current cancer therapeutics, indicating the requirement for novel more effective targets for cancer therapy [25]. Although the primary aim of chemotherapeutic drugs is to directly kill tumour cells, evidence has also demonstrated immune modulatory effects of some cancer therapeutics in patients with cancer. In addition, the potential selectiveness, reduced toxicity and immune enhancing properties by some natural product-based treatments for cancer have been highlighted [26, 27]. Hence, a recent growing interest in development of novel plant based cancer therapeutics with immune enhancing properties.

Cancer immunotherapy

Until recently, the contribution of the immune system towards tumour suppression was not appreciated, as it was first received with controversy due to lack of supporting evidence and limited experimental tools at the time. However in recent years, cancer immunotherapy has gained considerable interest and has provided new critical clues in cancer biology and research. Cancer immunotherapy is designed to harness and boost the host's natural immune response to recognise and eliminate cancer cells by intervening at different stages of the immune cycle [28]. The technologies are designed to promote immune-mediated killing of tumour cells by reversing the suppression by different immune cell phenotypes including tumour-specific T lymphocytes [29-31]. The aim is to activate and re-educate immune cells to recognise tumour cells and eliminate them, yet at the same time avoiding systemic over-activation related toxicity.

At present, various techniques utilized in cancer immunotherapy include those based on adoptive T cell transfer, monoclonal antibodies (mAb), cancer vaccination, oncolytic virus therapy and immune check point inhibitors [29-31]. Monoclonal antibody-

based treatments were first approved by the Food and Drug Administration (FDA, USA) agency in 1997 for Rituximab for the treatment of B-cell lymphomas [32]. The Rituximab monoclonal antibody (mAb) is designed to target the CD20 antigen abundantly expressed on B cells, to induce cell death via cell-mediated cytotoxicity [32]. Since the first therapeutic mAb approval, various mAbs have been approved including the anti-Her2 Trastuzumab for metastatic breast cancer, Alemtuzumab for B-Cell chronic lymphocytic leukaemia and Cetuximab for metastatic colorectal and head and neck carcinoma and many others [29, 33].

Cancer vaccination strategies, including dendritic cell-based cancer immunotherapies were also introduced in the mid-1990s. Dendritic cell-based vaccines are designed to exploit the intrinsic ability of dendritic cells to activate tumour specific effector cytotoxic T lymphocytes and natural killer cells against tumour cells, thereby eliminating malignant cells in the process [34, 35]. The immunotherapeutic technique involves *ex vivo* stimulation and activation of dendritic cells (DC), followed by injection of activated DCs carrying tumour associated antigens back into patients as therapeutic vaccines, with the aim of inducing attack of malignant cells and antitumor immunological memories [34, 35].

Immunological checkpoint blockade is also demonstrating significant success in cancer immunotherapy. The therapy utilizes immunomodulatory mAbs which target the surface receptors such as cytotoxic T-lymphocyte associated antigen 4 (CTLA-4) and the programmed cell death protein 1 pathway (PD-1/PD-L1), to block these immune checkpoint molecule receptors on T cells thereby inhibiting cancer-induced immunosuppression, leading to prolonged antitumor immune responses [36]. Currently FDA approved immune checkpoint inhibitors include Ipilimumab for melanoma treatment, Pembrolizumab and Nivolumab for unresectable metastatic malignant melanoma [33, 36].

Following promising results coming from the use of monoclonal antibodies, cancer vaccines and immunologic checkpoint inhibitors to target solid tumours, recently research has been expanding to exploit other potential immunologic targets. As stated earlier, the

tumour microenvironment is comprised of numerous types of immune cells that participate in the immune response and recently the significant impact of tumour associated macrophages (TAMs) and tumour associated neutrophils (TANs) in tumour angiogenesis have gained attention. Although most previous studies associated macrophages and neutrophils with promoting tumour growth, emerging evidence is highlighting the expanding functional roles of neutrophils, providing more strategic approaches that could be harnessed therapeutically.

Topic 2: Cancer immunology

The immune system and cancer

Tumour progression is determined by the interactions of a complex and dynamic network of malignant cells and communication mediators such as growth factors, cytokines, chemokines, inflammatory and matrix remodelling enzymes [1]. The interactions among these cells lead to tumour eradication, metastasis or establishment of dormant state. The immune system plays a significant role in cancer development and literature has shown that in addition to protecting the host against tumour formation, it also shapes tumour immunogenicity [1, 37]. In principle, the host's immune system should be able to recognise and eliminate nascent malignant cells in the same way that it protects the host against invasion by pathogens. However, tumours are able to exploit the regulatory system by hijacking and amplifying the activity of regulatory immune cells creating an immunosuppressive environment, avoiding recognition and destruction by the immune system [19, 29, 30].

The tumour immunoediting concept divides the events that lead to development of cancer into three sequential phases; the elimination phase, the equilibrium phase and the escape phase. The elimination phase is described as the first stage, where normal cells that have been transformed through exposure to harmful agents such as carcinogens, radiation, viral and bacterial infections leading to expression of neo-antigens and secretion of signalling molecules inducing an immune response [19]. In response, the innate and adaptive immune systems work in collaboration to eliminate tumour cells before they become clinically apparent [18, 19]. Incomplete elimination of tumour cells by the immune system leads to a state of equilibrium. The equilibrium phase is described as a stage where tumour cells that have managed to evade the elimination phase manage to progress. However, members of the adaptive immune system such as interleukin-12 (IL-12), interferon-gamma (IFN-γ), $CD4^+$ and $CD8^+$ T cells keep the residual tumour cells in a dormant functional state, preventing growth of tumour cells [18].

Nevertheless, due to the immune selection and genetic instability, the tumour cells in a dormant growth state are able to evolve by losing tumour expressing antigens, changing their genetic expression, thereby reducing recognition or are capable of increasing resistance towards the immune system, thereby leading to the escape phase [18, 19]. Evolved tumour cells continue to escape immune detection via over production of a range of chemokines and cytokines recruiting more immune suppressive cells progressively growing and creating an immune suppressive microenvironment to become less visible [18, 19]. Figure 1below by [18] illustrates the immune editing concept events.

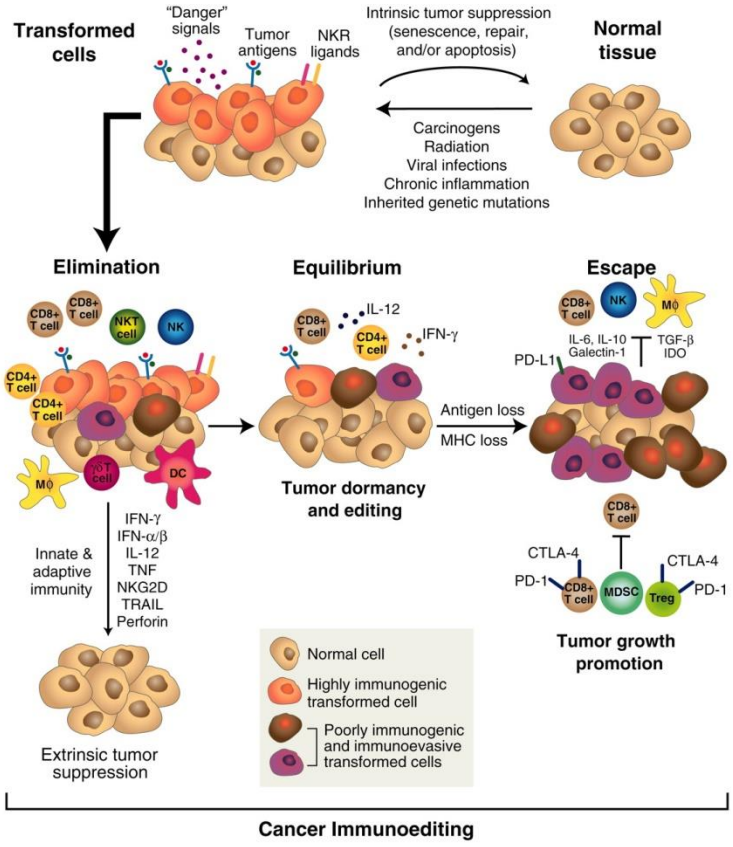


Figure 1 The immunoediting concept [18]

The tumour microenvironment

The tumour microenvironment consists of transformed malignant cells, with a supporting network of normal cells such as stromal fibroblasts, infiltrating immune cells, signalling molecules, soluble factors and extracellular matrix [38, 39]. As stated above, malignant cells have been known to manipulate normal cells, altering their functional mechanisms creating an environment which promotes tumour invasion and metastasis. Therefore, the tumour microenvironment is constantly evolving through remodelling of tissues, alterations of cancer metabolism and recruitment of various immune cell populations with different functional roles into tumours [39]. These constant changes in the biomechanical function of the tumour microenvironment contribute towards tumour progression, metastasis and secretion of tumour promoting immune mediators. For instance, altered tumour microenvironments which cause changes in vascular flow and interstitial fluid pressure, are associated with reduction of effective anticancer drug delivery [38]. Therefore, ongoing efforts have been focusing on therapeutic strategies which combine targeting of the tumour microenvironment and tumour cells at the same time, hoping the synergistic effect will help eradicate tumours [39].

Anticancer immune response

The immune system utilizes different mechanisms to prevent cancer development, including preventing viral infections and destroying the inflammatory causing pathogens thereby suppressing virus-induced tumours and inhibiting progression of an inflammatory environment that promotes tumour growth, respectively [18, 20]. Furthermore, a healthy immune system is able to recognize transformed cells which co-express ligands and antigens that are not expressed on normal cells, leading to elimination of precancerous and cancerous cells in tissues before they can progress [18]. Solid tumours are usually characterised by the dramatic expansion of various types of immune cells with different morphologies and phenotypes within and as such, the tumour microenvironment has been used as a therapeutic target for improved cancer drug development [37].

Although the main aim of conventional anticancer therapeutics is to directly destroy cancer cells, they may also induce the innate and adaptive immune responses to attack cancer cells. The contributions of immune cells in cancer therapy are paradoxical because they can either promote or prevent the potency of cancer therapeutics.

Cancer related inflammation

Various immune cells and mediators have been implicated in the enhancement of cancer related inflammation in the tumour microenvironment. Although inflammation is important for resolving infection due to pathogen invasion, chronic inflammation has been linked with stimulation of events and processes which can induce and support development of human cancers, including bladder, cervical, colorectal, gastric, liver, lung and pancreatic cancers [38, 40, 41]. Evidence shows that chronic inflammation triggers development of cancer through the extrinsic pathway (due to infection or autoimmune diseases) and the intrinsic pathway (due to genomic alterations) [42]. For example, infection with the *Helicobacter pylori* bacteria, hepatitis B or C virus and inflammatory bowel disease has been shown to induce chronic inflammation which enhances development of gastric cancer and gastric mucosal lymphoma, hepatocellular carcinoma and colon cancer, respectively [41, 42].

Members of the innate immune response such as granulocytes, macrophages, mast and neutrophils have been known to massively contribute towards chronic inflammation by producing chemokines, angiogenic growth factors and matrix degrading enzymes [38]. Myeloid lineage cells also produce DNA-damaging agents such as nitrogen species and reactive oxygen species (ROS), promoting genomic instability of the tumour [20, 38]. A range of cytokines and chemokines have been identified as cancer related inflammation key players, which include necrosis factor-kappa B (NK-κB), tumour necrosis factor (TNF), interlukin-6 (IL-6), cycloxygenase-2 (COX-2), chemokine CXC-receptor 4 (CXCR 4) and chemokine (C-X-C motif) ligand 8 (CXCL8) [40, 41]. Therefore, a range of anti-inflammatory drugs have been used to block key pro-inflammatory cytokines and chemokines in cancer therapy, including targets for COX-2, TNF, IL-6 and

CXCR4 in melanoma, osteosarcoma, breast and prostate tumours [40, 43]. However, they could be limited by development of unintended diseases and the risk of suppression or activation of other molecular processes. For instance, studies have shown that inhibition of the NF-κB (which has pro and anti-inflammatory functions) as a therapeutic target for autoimmune diseases and chronic inflammation related cancers, may also induce or elevate inflammation under certain conditions [39]. Hence, further investigations and understanding of mechanisms underlying immune mediated responses in cancer therapy are required, in order to come up with novel cancer therapies.

Immunosuppressive tumour microenvironment

Various immune cells and mediation factors are suggested to contribute towards tumour-induced immune suppression in animal models and patients with cancers. Tumour cells create an immune suppressive microenvironment via over production of cytokines such as transforming growth factor-β, galectin or indoleamine 2, 3-dioxygenase (IDO) and vascular endothelial growth factor (VEGF) [44, 45]. In response, the mediation factors recruit various regulatory immune cells such as myeloid-derived suppressor cells (MDSCs), tumour associated macrophages (TAMs) and regulatory T cells expressing forkhead box P3 (Foxp3Tregs) [44, 46]. Together these recruited cells work in collaboration suppressing antitumor immune responses, thereby allowing tumours to grow.

MDSCs are a heterogeneous population of immature myeloid progenitor cells widely present in most human cancers and experimental animal models and are perceived to play a critical role in the obstruction of the immune responses against tumours [45, 47]. MDSCs suppress cancer immune responses through various mechanisms, including disruption of T cell functions by up-regulating metabolic enzymes such as arginase 1 (ARG1), nitric oxide synthase 2 (NOS2) and IDO1, depleting critical metabolites required for lymphocytes functions [47]. Furthermore, MDSCs secrete immune suppressive molecules such as ROS, nitrogen species and anti-inflammatory

cytokines (interleukin 10 (IL-10) and transforming growth factor-beta (TGF-β), which activate generation of the anticancer immune suppressive Treg cells [45, 47, 48].

Tregs are specifically produced by the immune system to regulate self-tolerance and normal immune homeostasis [46, 49]. However, tumour cells can hijack their activity leading to inhibition of the function of antitumor effector cells, cytotoxic T lymphocytes (CTL) and their interaction with various antigen presenting cells (APCs) [18, 46]. Tregs highly express immunosuppressive cytokines such as TGF-β, IL-10 and negative costimulatory checkpoint inhibitory molecules CTLA-4, PD-1 and PD-L1 [46]. Due to their cancer promoting activity, increased Treg levels are associated with a poorer prognosis in patients with some cancers.

Adaptive anticancer immune response

As stated earlier, the immune system controls tumour progression via a coordinated process between the innate and adaptive immune responses. The adaptive immune system is comprised of immune cells such as $CD8^+$ cytotoxic T cells, $CD4^+$ helper T cells and B cells, which express somatically generated antigen-specific receptors that give them the ability to offer flexible and broad responses compared to innate immune cells [1, 49]. The functional behaviour of adaptive immune cells is highly dependent on the type of tumour, staging and the class of chemotherapy in use [49]. Although events that lead to recruitment of immune cells into the tumour microenvironment are yet to be fully understood, previous studies suggest that natural killers (NK) from the innate immune response are capable of recognising transformed cells through specific ligands expressed on tumour cells and destroy them [50]. During death tumour cells release protein molecules containing unique tumour associated antigens together with cytokines and other inflammatory signals that lead to activation of an inflammatory response [51]. In response, dendritic cells (DCs) take up these antigens, become activated and differentiate presenting these antigens to lymphoid cells such as cytotoxic $CD8^+$T cells via major histocompatibility complex I (MHC I) and major histocompatibility complex II (MHC II) molecules [34]. Activated $CD8^+$ T cells supported

by $CD4^+$ T helper 1 (TH1) cells which produce interleukin-2(IL-2) and interferon gamma (IFN-γ) traffic to the tumour site to attack and kill tumour cells leading to the release of even more tumour associated antigens, repeating the cycle [1, 51]. The activation of T and B cells, induce production of pro-inflammatory cytokines and chemokines, leading to further recruitment and activation of the innate immune cells such as macrophages and neutrophils into tumours, which in turn expand activation of tumour-specific T cell [50]. Although the adaptive immune response is slower to develop as compared to the innate immune response, they are known to generate tumour specific immune memories, preventing tumour recurrences.

Innate anticancer immune response

Members of the innate immune system are responsible for providing the host with the first line of defence against pathogen invasion and they are also abundantly found in tumours, which include neutrophils, dendritic cell (DCs), macrophages, natural killer cells, basophils, eosinophils, mast cells and several immune mediators [20, 49, 52]. Innate immune cells have the unique ability to respond promptly without previous assault memories or antigen specificity unlike adaptive immune cells. In the event of an infection or in cancer, sentinel cells like macrophages and mast cells release signalling molecules such as cytokines, chemokines and bioactive mediators recruiting additional leukocytes into damaged tissues inducing the inflammation process [1, 52].

The largest immune cell population within the tumours are the myeloid derived cells, which include DCs, macrophages and granulocytes, most with a morphology and phenotype similar to neutrophils yet are distinct in many of their biological characteristics and activities [51, 53]. As stated earlier, DCs play a critical role in the interface between innate and adaptive immune responses, by taking up and carry foreign antigens to lymphoid organs and present them to adaptive immune cells [34, 51, 52].

TAMs are a heterogeneous dominant leukocyte population largely involved in the tumour microenvironment, assuming a spectrum of roles required for tissue

homeostasis [2]. In early stages of cancer, macrophages of the M1 phenotype express high levels of IL-12, TNF-α and IL-6 which promote cytotoxic and inflammatory functions and in later stages of tumour growth macrophages assume an immunosuppressive M2 phenotype, which highly express IL-10, TGF-β and ARG1 cytokines [2, 48]. In essence, studies have indicated that TAMs respond to numerous factors in the tumour microenvironments which can influence their phenotype, similar to recent emerging evidence on neutrophils in cancer.

Neutrophils in cancer

Neutrophils are members of the innate immune system which are usually the first responders to sites of tissue damage and infection. They are considered the most abundant circulating leukocytes in humans making up between 50-70 % of the leukocytes population [54-56] . Activation of neutrophils is usually in response to bacterial or viral invasion and their primary role is to protect the host from damage and to provide recovery support of tissues after injury or inflammation [56-58]. Neutrophils use different mechanisms to eliminate host threats, including phagocytosis, secretion of pro-inflammatory chemokines and cytokines, release of cytotoxic chemicals from their intracellular granules, generation of reactive oxygen species (ROS) as well as formation of neutrophil extracellular traps (NETs) [54-57]. Although previously studies on the role of neutrophils in cancer were overshadowed by studies on specific T lymphocytes, recently neutrophils are increasingly recognised as important for their roles in tumour progression and regression.

Neutrophil production

Neutrophils are produced in the bone marrow via proliferation and differentiation of stem cells into mature neutrophils in a process called myelopoiesis [58]. The release of neutrophils from the bone marrow into the circulation is carefully regulated and maintained by chemokine interactions in order to maintain a balanced state. The retention of neutrophils in the bone marrow is regulated by the chemokine CXCR4, coordinated with its interaction with stromal derived factor-1 and chemokine C-X-C motif

ligand 12 (SDF1, CXCL12) [59-61]. The CXCL12 chemokine is largely expressed in the bone morrow, where it binds to the CXCR4 expressed on neutrophils leading to neutrophil homing [60]. Events of infection or tissue damage lead to activation of neutrophils by inflammatory mediators causing elevation of peripheral blood neutrophil counts. The down regulation of CXCR4 and CXCL12 is controlled by granulocyte-colony stimulating factor (G-CSF) leading to the release of neutrophils into circulation [60]. The Interleukin 8 receptor beta (CXCR2) cytokine and its ligands chemokine (C-X-C motif) ligand1 (CXCL1) and chemokine (C-X-C motif) ligand (CXCL2) are also suggested to be involved in controlling the release of neutrophils from the bone marrow, opposing the CXCR4 signalling antagonistically [60]. Figure 2 below represents processes and events which lead to the release of neutrophils into circulation.

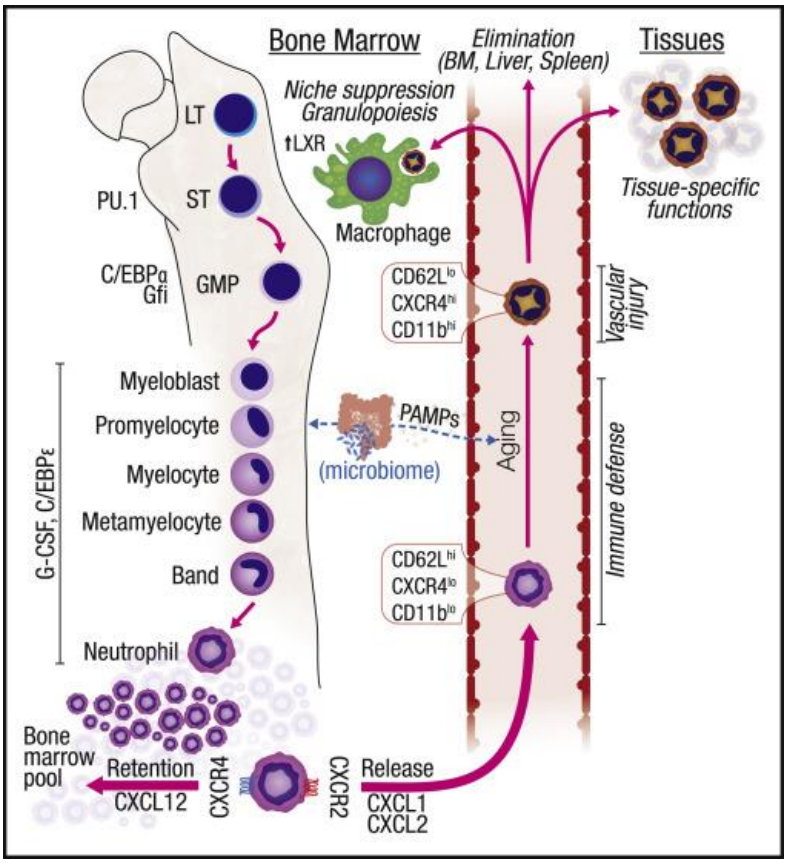


Figure 2: Schematic model of the neutrophil life cycle [62]

Tumour associated neutrophils

Neutrophils found in the tumour microenvironment are referred to as tumour associated neutrophils (TANs). In humans TANs are identified as $CD66b^{+}$ cells and in

murine tissue as $CD11b^{+}Ly6G^{hi}$ cells [63-66]. The role of neutrophils in cancer is surrounded by controversy and is yet to be fully understood. Previous studies have extensively reported on the role of neutrophils in promoting tumour angiogenesis, invasion and metastasis [4, 37, 67-69]. Conversely, recent studies have also linked neutrophil presence with inhibition of tumour growth [5, 70].

Neutrophils are primarily the key players during the inflammatory responses and chronic inflammation has been associated with cancer promotion. As a result, a high neutrophil-to-lymphocyte ratio (NLR) is associated with poor prognosis in some cancer types and therefore, NLR is used to predict clinical patient outcomes [49, 71]. However, the prognostic value and impact of neutrophils (high or low) differs among cancers or even among patients. These observations raise the question of whether neutrophils are recruited for a specific role in tumours or whether different stimuli induce mobilization of specific phenotypes.

Morphology and surface markers of tumour associated neutrophils

Traditionally neutrophils are characterised as myeloid cells identified by a specific nuclear morphology, granule content and a short half-life, although recent evidence has highlighted the heterogeneity of neutrophils in cancer [6, 58, 72]. In murine tissues, neutrophil surface expression markers include glycosylphosphatidylinositol (GPI)-linked receptor (Ly6G) recognized by mAb clone 1A8, positive staining for myeloperoxidase and absence of F4/80 (monocyte/macrophage marker) and in humans, the expression of CD66b [65, 73]. In recent studies, several neutrophil subpopulations have been identified and defined by a combination of features which include density, maturity, surface markers, morphology and anatomical site as discussed below.

Continuous density gradient techniques such as Ficoll-Hypaque and Histopaque density 1.119 and 1.077 g/mL have been used in several studies to isolate neutrophils from peripheral blood and tissue samples for functional and nuclear morphological studies [72, 74, 75]. The technique can separate neutrophils based on density, allowing purification of high density neutrophils (HDNs) and low-density neutrophils (LDNs).

Following density gradient separation, distinct LDN and HDN neutrophil subsets demonstrating opposing cancer related functions have been isolated and identified from whole blood of murine tumour models or blood collected from human patients with either lung or breast cancer [72]. Furthermore, another study indicated that mice bone marrow neutrophils differentiate into a unique hybrid phenotype with dual properties of both neutrophils and DCs, and which stimulate differentiation and activation of CD4 T cells [8]. Unfortunately, the heterogeneity of circulating neutrophils in cancer poses a major challenge in their identification and characterization at present; due to lack of distinctive membrane bound markers among diverse subpopulations.

Neutrophil recruitment and activation

The knowledge of how neutrophils interact with other immune cells and their recruitment to infection sites or damaged tissue has been extensively expanding over the years. As mentioned earlier, trafficking of TANs into inflammatory sites begins with their differentiation in the bone marrow under the influence of haematopoietic growth factors, G-CSF and granulocyte/monocyte colony-stimulating factor (GM-CSF) [76]. Migration of neutrophils to sites of infection is influenced by specific chemokines, cytokines and cell adhesion molecules located on their surfaces and on endothelial cell surfaces. Upon release from the bone marrow, circulating neutrophils are mobilized into inflammatory sites by the “danger” chemical signals produced by damaged tissues. Tumours also secrete chemotactic substances such as interleukin-8 (IL-8) and G-CSF, skewing the amounts of blood neutrophils [57]. At infection sites, activated neutrophils release cytotoxic proteinases damaging surrounding tissue and they also produce chemokines and cytokines recruiting even more immune cells [60].

The cellular-mesenchymal-epithelial transition factor (c-MET), a surface receptor and its ligand, hepatocyte growth factor (HGF), have also been associated with the chemoattraction and antitumor cytotoxicity of neutrophils [17]. Expression of the c-MET proto-oncogene results in stimulating numerous cellular processes such as the cell cycle, proliferation, differentiation and motility of cells. However, HGF signalling has also

been associated with the pathogenesis of some aggressive and metastatic cancers [77, 78]. The receptor molecule c-Met is also expressed on TANs and, it was indicated to respond to inflammatory signals (which can also be tumour derived) through the action of tumour necrosis factor (TNF)-α leading to activation and recruitment of antitumor cytotoxic neutrophils to tumour sites [17]. More so, a recent study also indicated TNF as a key cytokine in mediating neutrophil cytotoxic activity in patients with breast cancer and treatment of neutrophils from healthy breast cancer control patients with cytokines increased the anticancer neutrophil cytotoxicity [79].

Although the antitumor cytotoxic nature of neutrophils was suggested more than three decades ago, there have not been many studies pursuing this line of research until recently [80, 81]. Emerging evidence has shown that neutrophils are capable of differentiating into multiple phenotypes but, little is known regarding the induction processes and mechanisms that lead to this development. The concept that neutrophils have a double role (pro- and anti-tumorigenic) in the regulation of responses in cancer was highlighted by Fridlender et al, 2009, when their group indicated the polarization of tumour associated phenotypes by the cytokine transforming growth factor beta (TGF-β) and defined them as either N1 or N2 [6]. According to Fridlender and colleagues, differentiation of neutrophils is regulated by TGF-β and interferon beta (IFN-β) and the presence of TGF-β, promotes differentiation of TANs into the low activation state, phenotype N2 which supports tumour growth through secretion of protumour growth factors, enhancement of angiogenesis, increased degradation of the extracellular matrix and immunosuppression [6]. Conversely, blocking of the TGF-β and the presence of IFN-β promotes differentiation of TANs into a high activation state phenotype N1, which exhibit increased antitumor cytotoxicity, enhanced expression of immune activating chemokines and cytokines and the ability to activate the adaptive immune response [6].Figure 3 below demonstrates the origin and fate of TANs in cancer. Recently, further studies have indicated the antitumor cytotoxicity of neutrophils [5, 17, 64, 70, 72, 79, 82]. In animal models tumour entrained neutrophils blocked metastatic seeding in the lungs

via hydrogen peroxide mediated cytotoxicity [5]. Furthermore, TANs enhanced T cell activation in early-stage human lung cancer, through the cross-talk initiated by direct contact between activated TANs and T cells [70].

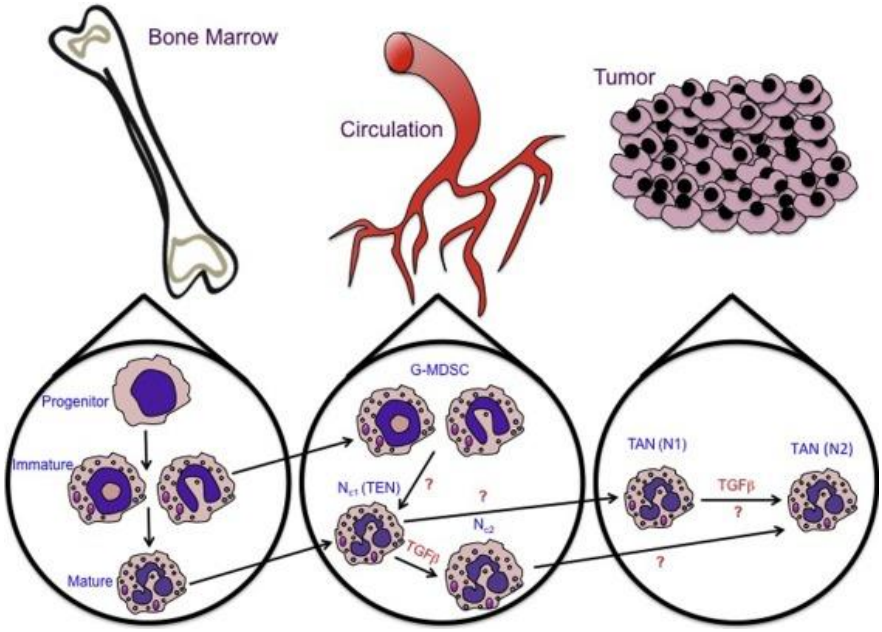


Figure 3: Schematic model on the origin and fate of circulating neutrophil subpopulations in cancer [63].

Neutrophil anticancer cytotoxicity

Since the primary function of neutrophils is to kill pathogens, it is not surprising that most of the antimicrobials they produce are harmless towards eukaryotic cells. However, activated neutrophils exert their cancer killing effects through controlled mechanisms using an array of toxic substances such as release of the NADPH oxidase complex, superoxides, hydrogen peroxide (H2O2), and hypochlorous acid (HOCl) [80, 83]. Neutrophil phagolysosomes contain enzymes (e.g. NADPH oxidase) which are capable of reducing molecular oxygen into ROS. In cancer, over production of ROS associated with infiltrating neutrophils has been reported to exert cytotoxic effects leading to tumour regression via direct disruption of tumour cell membranes, yet at the same time causing host tissue injury and initiation of DNA damage leading to mutations and tumour establishment [62, 83]. Furthermore, neutrophils have intracellular granules filled with azurophilic granules (peroxidase-positive), which contain myeloperoxidase (MPO), the oxidative burst enzyme which catalyses the conversion of H_2O_2 into the highly antiseptic

HOCL [73, 80, 84]. Activation of neutrophils in response to different stimuli, also lead to the release of NETs, which are extracellular fibrous structures assembled from granule proteins (elastase peptides and myeloperoxidase enzymes) attached to a structural backbone made of DNA chromatin and histones, which can trap and kill pathogens or damage surrounding tissue [55, 85].

The life cycle of neutrophils

Traditionally, neutrophils are known to be short-lived (under 24 h *ex vivo*) although emerging evidence suggests otherwise and with some findings debatable [86]. For example, oral administration of neutrophils labelled with $^{2}H_2O$ in mice and humans studies, showed an average circulatory neutrophil lifespan of 12.5 h and 5.4 days, respectively [87]. However, the following year an article was released objecting and raising concerns over the findings [88].

Nevertheless, some studies suggest that the neutrophil life span vary and can be extended by pro-inflammatory stimuli, although under normal conditions neutrophils die by apoptosis to maintain homeostasis. However, during serious infection some neutrophils undergo other styles of deaths including necrosis, autophagy and the unique NETosis death [89]. Literature shows that some obligate bacteria, viruses, fungi and parasites are capable of delaying the neutrophil apoptosis death for their own beneficial gains, by creating conditions which extend viability of their replicative niche [86]. These obligate pathogens extend the neutrophil life span by altering the expression of genes associated with apoptosis, down regulating pro-apoptotic factors, thereby blocking activation of NADPH oxidase and preserving mitochondrial integrity causing the delay of caspase-3 activation [86].

Neutrophil Cell death

Apoptosis

Death of cells is usually important for normal development and maturation cycle, maintaining adult tissue homeostasis [90, 91]. Traditionally, mechanisms of cell death have been divided into apoptosis, commonly referred to as type I programmed cell death and necrosis [91]. However, neutrophil cell death is further divided into autophagy (type II

programmed cell death) and NETosis [89] The apoptosis cell death mechanism represents an energy-requiring spontaneous single cell death, which is characterised by a series of molecular, biochemical and morphological changes and Annexin V stain is commonly used to detect externalization of cell membrane phosphatidylserines, as a marker for apoptosis [90]. The process is induced by the intrinsic (triggered by cellular stresses due to DNA damage, activated oncogenes, hypoxia, oxidative stress and irradiation) or extrinsic (signalling through TNF receptors) death signalling [90]. The morphological changes can be identified by cell contraction, dense cytoplasm and densely packed organelles under the light microscope.

Necrosis

Regulation of neutrophil cell death by apoptosis is critical for resolution of inflammation, as it prevents deployment of cytotoxic intracellular components into the surrounding environment and reduces production of pro-inflammatory cytokines [86]. In contrast to apoptosis is the necrosis cell death pathway, which is usually identified and characterised by swelling of the cell, formation of cytoplasmic vacuoles and blebs, swollen or ruptured mitochondria, disrupted membranes and eventually explosion releasing cytoplasmic contents into local tissue environments [90]. Necrosis is regarded as toxic, since the release of proteolytic enzymes and oxidant generating enzymes (cytoplasmic contents) lead to tissue injury and activation of pro-inflammatory signals which recruit inflammatory immune cells and other effectors [91].

Autophagy

Autophagy also referred to as programmed cell death II, represents an important cellular catabolic degradation response to diverse stresses and signals, to maintain intracellular metabolic homeostasis (conserve cell life) [89, 92, 93]. The process is characterised by formation of autophagosomes, aggregated proteins and organelles, destined for recycling of cytoplasmic components [89, 92]. The main purpose of autophagy death is to prolong cell survival under extreme conditions and is considered to not activate inflammatory responses [89, 93].

NETosis

NETosis is a process of cell death only specific for neutrophils, which lead to formation of extracellular NETs [89]. As mentioned a, NETs are DNA based structures containing MPO granules and proteases molecules which they use to immobilise microbes facilitating the subsequent phagocytosis process [94, 95]. Activation of neutrophils by different stimuli initiates the formation of NETs, leading to migration of neutrophil elastase and myeloperoxidase from granules to the nucleus, where they mix with nuclear chromatin eventually forming functional NETs [89]. The releasing of nuclear DNA and granule proteins in NETs create a high local toxic concentration which damages surrounding tissue or pathogens and the processes is characterised by NADPH oxidase activity, disintegration of the nuclear envelope and granule membranes [89, 94]. A previous study showed that NETs appear like debris of the dead cells as they are hardly visible on light microscope [89].

Topic 3: Natural product-based treatment for cancer

Natural products and their derivatives have been used as a source of medicinal products in almost all human cultures throughout history because they offer a broad chemical diversity of therapeutic compounds. Although we have witnessed a tremendous improvement in the modern pharmaceutical industry, about 75 % of cancer therapeutics are originally derived from natural products [96]. Moreover, these chemical structures are still used as model designs for synthetic anticancer therapeutics [97]. Anticancer therapeutic agents extracted from natural products remains attractive when compared to synthetic compounds because they tend to exhibit reduced toxicity and more structural diversity [27, 98].

The naturally derived cancer therapeutics includes vinca alkaloids, taxanes, tubulin-binding agents, anthracyclines and epipodophyllotoxins [98, 99]. These compounds exert anticancer effects through various mechanisms such as induction of apoptosis, inhibition of protein synthesis and angiogenesis as well as disruption of DNA structures leading to cell death [99]. To date, several plant derived anticancer compounds have been approved for use including vinblastine for Hodgkin's lymphoma, camptothecin for recurrent colon cancer, taxol and naveilbine [98]. Natural plant based anticancer or chemopreventative products derived from essential oils, fruits and leaves have gained interest in recent years [96, 100]. For example, Ingenol mebutane (marketed as Picato) extracted from the leaves of the Australian plant *Euphorbia peplus,* was approved for use in Australia in 2013, as topical treatment for actinic keratosis (AK) and has been showing promising results [101, 102]. Although previously anticancer compounds were designed to target specific cell structures or molecules, studies have also indicated immunomodulatory activities by some natural anticancer compounds including the essential oils [103, 104].

Essential oils extracted from aromatic plants such as tea tree oil, jasmine and lavender have been shown to have antimicrobial, anti-inflammatory and anticancer

properties [105, 106]. Phytochemicals such as essential oils have been shown to exert anticancer effects through various mechanisms, including by functioning as antimutagenic, antiproliferative as well as antioxidants preventing oxidative damage [103, 105]. Anticancer compounds with immunomodulatory properties exert anticancer effects by suppressing or disrupting specific inflammatory responses or molecular pathways inhibiting tumour growth [104]. Essential oils with anti-inflammatory properties can work as antioxidants through inhibition of cell toxic damage during mild redox stress by detoxifying excessive ROS [104]. Also, studies have reported anticancer effects of monoterpenes compounds derived from essential oils in various human cancer cells including leukaemia, glioma, colon cancer, gastric cancer and human liver tumours [105-107]. Tea tree oil (TTO), an essential oil extracted by steam distillation from leaves of the *Melaleuca alternifolia* plant is one of the essential oils which has gained attention as an anticancer, viral, fungal and antimicrobial compound over the years [11, 107-109].

Melaleuca alternifolia

Melaleuca alternifolia is an Australian native plant with a long history of medicinal use, including for wounds and cutaneous infections amongst the Aboriginal communities [109, 110]. *Melaleuca alternifolia* extracts mainly consist of monoterpenes, sesquiterpenes and many other constituencies although terpinen-4-ol is considered as the main bioactive compound [107, 110]. The anticancer effects of terpinen-4-ol and other TTO extracts have been reported in several studies, including human melanoma cells *in vitro*, human non-small cell lung cancer *in vitro* and in various murine animal models studies [12, 14, 15, 111]. Additionally, TTO impaired growth of human breast cancer MCF-7 cells and mouse breast cancer 4T1 cells, while no indication of cytotoxicity was demonstrated on fibroblasts (HFF-1) and peripheral blood mononuclear cells (PBMCs) as demonstrated in Figure 4 [26].

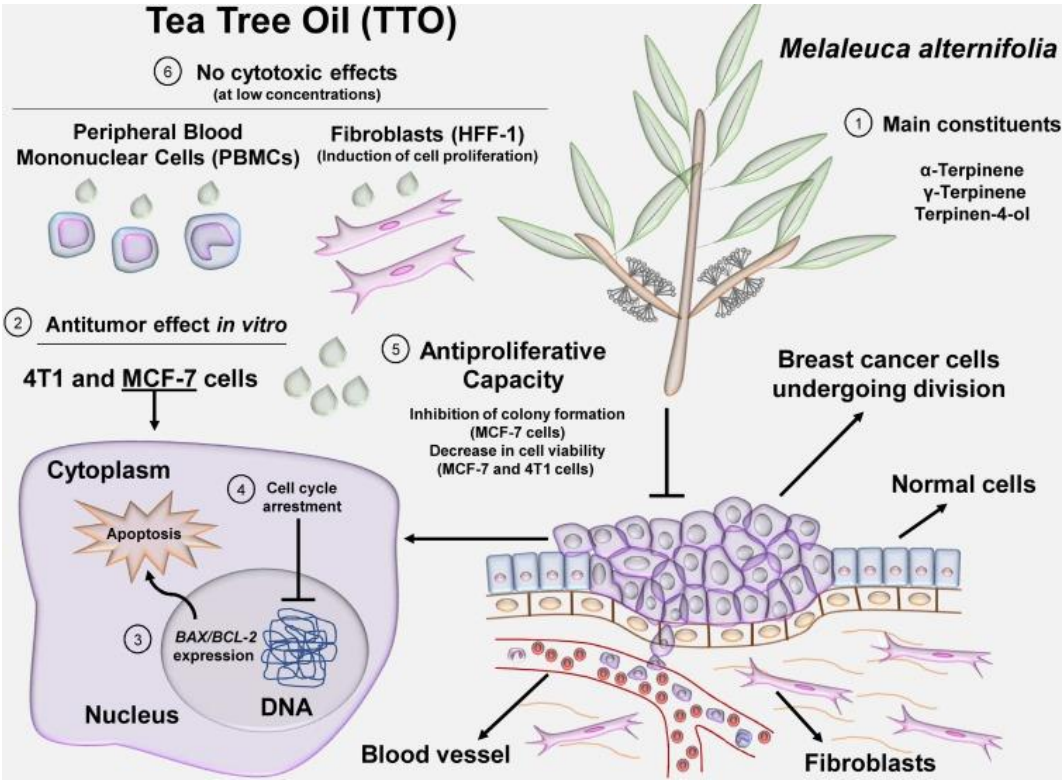


Figure 4: Anticancer effects of TTO.
TTO has several constituents such as α-Terpinene, γ-Terpinene and Terpinen-4-ol, potentially involved in the observed responses; 2) TTO presents antitumor activity *in vitro* on MCF-7 breast cancer cells by mechanisms related to 3) expression of genes involved with programmed cell death, such as *BAX/BCL*-2, 4) cell cycle arrestment and 5) antiproliferative activity by inhibiting colony formation and decreasing cell viability of MCF-7 and 4T1 cells; 6) TTO has no cytotoxic effect on fibroblasts and PBMCs at low concentrations, increasing cell proliferation of fibroblasts [26].

Melaleuca Alternifolia Concentrate

Melaleuca Alternifolia Concentrate (MAC) is a complex TTO derived compound invented by Prof. Max Reynolds (ex-Griffith University) and was supplied by 98 Alive Pty Ltd (Australia). MAC is a heterogeneous mixture of mostly monoterpenoids and sesquiterpenes, acquired by further refining; removing the toxic constituents associated with irritations and the allergic reactions that are commonly associated with other TTO extracts [14, 112]. The more highly purified composition of MAC is suggested to increase its potency while reducing adverse side effects, making the compound more selective [14]. Table 1 below, demonstrates a comparison between MAC compound and standard TTO listing their main constituents.

Table 1: MAC composition compared to standard TTO compound

Table 1.2: GCMS chromatographic profile for MAC

Components	TTO ISO 4730 Int. Std. (%)[1]	MAC (%)[2]	Molecular Weight	Terpene type
Terpinen-4-ol	>30.0	60.0 – 64.0	154.2	Monoterpenoid
p-Cymene	0.5 – 8.0	8.0 – 14.0	134.2	Monoterpene
α-Terpineol	1.5 – 8.0	5.0 – 7.0	154.3	Monoterpenoid
δ-Cadinene	Trace – 8.0	4.0 – 6.0	204.4	Sesquiterpene
Aromadendrene	Trace – 7.0	0.5 – 1.5	204.4	Sesquiterpene
1,8-Cineole	Trace – 15.0	0.8 – 1.2	154.3	Monoterpenoid
γ-Terpinene	10.0 – 28.0	0.5 – 1.0	136.2	Monoterpene
Terpinolene	1.5 – 5.0	0.2 – 0.5	136.2	Monoterpene
Globulol	Trace – 3.0	0.3 – 0.4	222.4	Sesquiterpenoid
Ledene	Trace – 7.0	N/A	204.4	Sesquiterpene
Viridiflorol	Trace – 1.5	N/A	222.4	Sesquiterpenoid
α-Pinene	1.0 – 6.0	N/A	136.2	Monoterpene
Sabinene	Trace – 3.5	N/A	136.2	Monoterpene
α-Terpinene	5.0 – 13.0	N/A	136.2	Monoterpene
Limonene	0.5 – 1.5	N/A	136.2	Monoterpene

[1] (International Standards Organisation, 2004)
[2] (Reynolds, 2006, 2009a, 2010)

Although most of the studies of MAC are still unpublished, MAC elicited cell death both *in vitro* and *in vivo* via the intrinsic mitochondrial pathway [14, 112]. Furthermore, MAC retarded growth of subcutaneous tumours in murine breast cancer models accompanied with an immunological response in the transgenic FVB/N c-neu spontaneous murine breast cancer model alone, indicating that the immunological response was model based. [14]. Tumour regressions in FVB/N c-neu models were associated with an accumulation of neutrophils inside tumours. Hence, further immunological studies were required to elucidate whether tumour infiltration with neutrophils also mediated the eradication of residual tumour cells. In a follow up study, MAC was shown to induce an immunological response through blockade of lipopolysaccharide induced NF-κB signalling activation preventing production of inflammatory cytokines in macrophage-like cell lines [112]. Recently, increasing evidence has been reported showing the heterogeneity of neutrophils in cancer (as described in sections above) although, the role of neutrophils in promoting tumour regression is not yet well characterised. Therefore, this study was aimed at identifying, characterizing and

investigating the specific roles played by the activated tumour infiltrating neutrophils in response to treatment of murine breast cancer models with MAC.

Hypothesis and aims

H_0 Injection of MAC intratumourally into subcutaneous tumours of transgenic FVB/N neu spontaneous murine breast cancer models induce an immunological response against the tumour cells.

H_1 Injection of MAC intratumourally into subcutaneous tumours of transgenic FVB/N neu spontaneous murine breast cancer models have no immunological effect against the tumour cells.

H_0 TANs activated in response to MAC treatment have a hypersegmented nuclear morphology typical of polymorphonuclear neutrophils.

H_1 TANs activated in response to MAC treatment have a normal nuclear morphology typical of neutrophils.

H_0 *A subtype of TANs activated in response to MAC treatment is cytotoxic for tumour cells.*

H_1 A subtype of TANs activated in response to MAC treatment has no cytotoxic towards tumour cells.

Chapter 4 aimed at investigating the immunomodulatory activity of MAC *in vivo* and the potential anticancer cytotoxicity of neutrophils *ex vivo*. The potential immune response induced by MAC was investigated *in vivo* using female transgenic FVB/N c-neu spontaneous murine breast cancer as a model system. Changes in neutrophil populations in the tumour microenvironment were analysed by flow cytometry. Furthermore, the morphological characteristics of isolated TANS were analysed by immunocytochemistry and the potential cytotoxicity of the purified neutrophils against NeuTL cells were analysed *ex vivo*.

Chapter 3: Materials and Methods

MATERIALS AND REAGENTS

Table 2: Mammalian cell lines

Cell line	Description	Supplier/ Obtained from	Growth Media
NeuTL	Spontaneous murine breast cancer	Laboratory stock	DMEM

Table 3: Materials for mammalian cell culture

Product	Cat. No.	Company
Dulbecco's Modified Eagle's Medium (DMEM)	10313-021	ThermoFisher Scientific
Penicillin and streptomycin solution	P4333	Sigma-Aldrich
GlutaMAX	11574466	ThermoFisher Scientific
Fetal bovine calf serum (FBS)	12103C	Sigma-Aldrich
β-mercaptoethanol	01496DK	Sigma-Aldrich
4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	H4034	Sigma Aldrich
Trypan blue solution 0.4 %	T8154	Sigma-Aldrich
Trypsin-EDTA	59417C	Sigma-Aldrich

Table 4: Materials and reagent for cytotoxicity studies

Product	Cat. No.	Company
Tergitol NP-9	17881	Fluka
SYTOX Green	S7020	ThermoFisher Scientific
DMEM, phenol red free	21063029	ThermoFisher Scientific

Table 5: materials and reagents for cell biological mechanism-based assays

Product	Cat. No.	Company
Hoescht 33258	H3570	Sigma
Histopaque 1.119	RNBG0646	Sigma
Histopaque 1.077	RNG5100	Sigma

Table 6: materials and reagents for murine treatment

Product	Cat. No.	Company
Female transgenic FVB/N c-neu mice	N/A	Griffith University animal facility
MAC oil	Batch MJR-1-2	98 Alive Pvt Ltd
D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS)	BCBT2435	Sigma
Manual Callipers	N/A	Lab stock
1 mL graduated syringe 27 and 30 gauge needle	N/A	Lab stock
Shaver	N/A	Griffith University animal facility

Table 7: Materials and reagents for immunocytochemistry

Product	Cat. No.	Company
Hematoxylin solution Gill No.3	121M4355	Sigma
Eosin	N/A	Sigma
Methylene Blue powder	N/A	Laboratory stock
Slides	N/A	ThermoFisher Scientific

Table 8: Antibodies for flow cytometry

Antibody	Fluorophore	Clone	Host	Cat. No	Company
Fixable Viability Dye	eFluor 780	N/A	Rat	65-0865	eBioscience
CD45	Super Bright 600	30-F11	Rat	63-0451-82	eBioscience
Mouse CD11b	PerCP-Cy5.5	M/70	Rat		eBioscience
Mouse Ly6G	Pacific Blue	1A8	Rat	60031PB	STEMCELL Technologies
Mouse Ly6G	Horizon V450	1A8	Rat	56-0603	BD Pharmingen
C-MET	FITC	eBioclone 7	Rat	11-8854-82	eBioscience
UltraComp eBeads	N/A	N/A	Rat	01-2222-41	eBioscience

Preparation of reagents

Preparation of MAC

MAC was supplied by 98 Alive Pty Ltd (Australian Botanical Bioscience Pty). MAC is a lipophilic mix of compounds and hence an emulsifier, D-alpha Tocopherol polyethylene glycol succinate (TPGS) was used as a vehicle to help dissolve MAC into the culture media and for injections. Previous studies have shown reduced tissue penetration when *Melaleuca Alternifolia* oil or terpinen-4-ol are administered alone and hence, common dissolving agents such as DMSO, TWEEN 20 and TPGS have been used in previous studies [15]. TPGS was selected as a solubilizing agent because it is approved by the FDA as a vehicle for drug delivery, as well as for a water soluble vitamin E nutritional supplement [113, 114].

TPGS

TPGS in the form of a white wax was acquired from SIGMA Aldrich. A 2 % (wt.) stock TPGS aqueous solution was prepared by melting 0.5 g of TPGS wax at 37-41 ^{o}C on a hot plate. 24.5 mL of hot MilliQ water was added slowly, continuously stirring with a magnetic stirrer. The mixture was continuously stirred for 2 h until colourless, homogenous and allowed to cool before being filtered and stored at room temperature. Working solutions of 0.5 % TPGS (w/v) were prepared by further dilutions using PBS 1x.

Compound solubilisation

For *in vivo* studies, 4 % v/v MAC solutions for injections were prepared by dissolving MAC oil in 0.5 % w/v TPGS as vehicle the day before each treatment cycle began and was stored under dark conditions at room temperature. TPGS (0.5 % alone) was used for vehicle control injections.

Preparation of flow cytometry staining buffer

Flow cytometry 2 % FBS/PBS 1x staining buffer was prepared by adding 10 mL of heat inactivated FBS to 490 mL of PBS 1x. The solution was thoroughly mixed and kept on ice or 4^{o}C for no longer than 3 days.

Preparation of 4 % Formaldehyde solution for cell fixation

4 % formaldehyde was prepared from paraformaldehyde powder acquired from Sigma Aldrich and the preparation procedure was carried out in a ventilated hood. To prepare 100 mL of 4 % formaldehyde, 80 mL of PBS 1x was heat to approximately 60 ^{o}C in a beaker on a stir plate. 4 g of the paraformaldehyde was added gradually, stirring continuously adding a few drops of 1 M NaOH to raise the solution pH. Once dissolved, the solution was cooled, adjusted the volume to 100 mL using PBS 1x and the pH of the solution to approximately 6.9 using dilute HCL. The prepared 4 % formaldehyde solution was filtered, kept in the dark at 4 ^{o}C until further use.

Preparation of Methylene blue solution for immunocytochemistry

0.1 % Methylene blue stain solution was prepared from laboratory powder stock. 0.1 g of the stock powder was weighed and dissolved in 30 mL of 95 % ethanol. The prepared solution was further diluted using 100 mL distilled water with 2 drops of KOH. The prepared stain solution was filtered using Whatman filter paper and stored in the dark at room temperature.

Anticancer and immunomodulatory activity of MAC in pre-clinical murine breast cancer model

In vitro Analysis

Mammalian cell culture

NeuTL cells

The NeuTL cell line was used for *in vitro* analyses in this study. NeuTL cells were originally derived from spontaneous tumours of the transgenic FVB/N c-neu spontaneous breast cancer model.

In vitro assays

All tissue culture preparations and cell passaging were carried out under sterile conditions in a Class II Biological Safety Cabinet (BSC II; BH200 series, Clyde-APAC). Neu TL cells were cultured in complete DMEM media supplemented with heat inactivated 10 % FBS, 1 % penicillin/streptomycin, HEPES buffer (25 mM pH 7.4), D-glucose (4.5 g/L), L-glutamine (584 mg/l), sodium pyruvate (110 mg/l) and β-mercaptoethanol (0.05 mM). DMEM media and the supplements were supplied from Invitrogen and the Neu TL cell line was obtained from the Griffith University laboratory stocks. NeuTL cells were grown in monolayers to 70-80 % confluency in 25 T or 75 T tissue culture flasks and kept at 37 ^{o}C in a humidified 5 % CO_2 incubator (HERAcell 150i, Thermo Scientific). Monolayers of cells were passaged every 3 to 4 days by treatment with 0.05 % trypsin-ethylenediaminetetraacetic acid (EDTA), washed with warm complete DMEM media, centrifuged at 1250 rpm for 5 min and resuspended in DMEM media.

Cell counting

To determine viability of cells, the trypan blue exclusion test was used. Harvested single cell suspensions were diluted in equal volumes of trypan blue solution (10 µL: 10 µL), then mixed gently. 10 µL of stained cells was transferred to a hemocytometer; dead and live cell numbers were counted using a light microscope. The average count from 5 squares using the equation below was used to calculate cell concentrations.

Equation1:

Total cell number =$average\ cell\ number\ x\ 2\ x\ 10\ 000\ x\ Total\ volume\ of\ media(mL)$

Cell number and plating

The optimal cell seeding density was determined by seeding NeuTL cells in a 96 well plate in accumulative densities (from 1000-5000 cells per well). Cell growth was observed at 24, 48 and 72h for monitoring cell confluency. 4000 cells per well was determined as the optimum seeding density for NeuTL murine breast cancer cells.

Table 9: Cell culture

Type	Cell line	Cell type	Cells/well
Malignant	NeuTL	Murine breast cancer	4000

Cells grown to 70-80 % confluency were harvested using the cell passaging method described above. Cell pellets were resuspended in phenol red-free DMEM media, counted and diluted using Equation 2 below. 100µL of cells were seeded using a multi-channel pipette into black well clear bottom Costar 96-well plates, incubated at 37ºC, 5 % CO_2 for 24 h.

Equation 2:

Dilution Factor =$\frac{Avearge\ cell\ number\ x\ 2\ x\ 10\ 000}{Cells\ per\ well\ x\ 100}$

Mycoplasma detection test

NeuTL cells were cultured to medium confluency on a glass coverslip sitting at the bottom of a tissue culture dish. Cells were washed with PBS1x, fixed with 4 % formaldehyde then washed again with PBS1x. Cells were stained with Hoechst 33358 stain (1mg/mL), incubated for 10 min in a dark place. After washing cells twice with PBS1x, cells on the cover slip were placed on a glass microscope slide and analysed under a fluorescent microscope with a 100x oil immersion objective.

In vivo studies

Animal resources, conditions and ethical approval

All animal work and studies were performed following the guidelines of The National Health and Medical Council of Australia (NHMCA), with the protocol approval from the Griffith University Ethics Committee (Ethics approval number MSC/13/16AEC). The female transgenic FVB/N *c-neu* spontaneous murine breast cancer model was used during the study. All mice were bred and housed with controlled light, temperature and humidity in the Animal Facility located at Griffith University (Southport, Australia).

Animal studies

The FVB/N c-neu strain of mice was genetically modified through over expression of the *Her2/neu* oncogene consisting of the mouse mammary tumour virus (MMTV) promoter driving a rat Erbb2 (Her-2) cDNA sequence [115]. Upon reaching puberty from about 4-8 months, 70 % of females develop breast cancer very similar to human Ductal Carcinoma In Situ (DCIS), a common form of human breast cancer. Mice from the age of 6 months were monitored every week for spontaneous tumour development and treatment initiation was started when tumours were approximately 250-350 mm^3 in size. Alternatively, female FVB/N c-neu mice showing no signs of growing spontaneous tumours were administered with a subcutaneous injection of 1x 10^6 Neu TL cells (a cell line derived from the transgenic FVB/N *c-neu* mice) on the right or left rear flank to initiate tumour growth in a more localised manner, to ensure tumours were obtained within a shorter time frame (2-3 weeks). Tumour sizes were monitored and measured using manual callipers and mice weights measured individually before treatment initiation, during and at treatment endpoint before mice were culled.

Establishment of subcutaneous tumours

NeuTL cell injection trial 1

To facilitate establishment of subcutaneous tumours, pilot studies were designed and performed to determine the optimum number of cells for injections. For the first pilot study, 3 FVB/n mice were used. Neu TL cells were grown to 60-70 % confluency, trypsinized using 0.05 % trypsin, resuspended in complete DMEM, counted and washed

twice in PBS 1x. Cells were resuspended in PBS 1x at $1x10^6$ cells in 100 µL for injection and kept on ice.

It should be noted that during cell culture, the Neu TL cells were observed to lift from the bottom of culture flasks when they had reached about 70 % confluency. A mycoplasma analysis was performed using Hoechst stain and the cells were determined to be mycoplasma free. Mice were shaved at the injection sites, sprayed with 80 % ethanol and the cell preparation were thoroughly mixed again and injected subcutaneously using a 1 mL graduated syringe with a 27 gauge needle.

Mice were monitored every day for general health check-ups, bruises or any reactions. Tumours were expected to appear 2-3 weeks post cell injections. At 30days post subcutaneous NeuTL cell injections (1×10^6 in 100µL PBS 1x) no tumour growth was observed in all 3 mice. The delay in tumour development was suggested to have been caused by either development of an immune response against the cells or use of unhealthy cells.

NeuTL cell injection trial 2

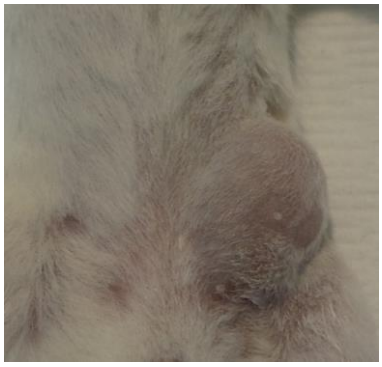
Another group of 3 mice were injected with a higher concentration of cells ($2x\ 10^6$ cells in 200uL PBS1X) using the same preparation and treatment conditions. On day 15 post cell injections, one mouse was observed to have a palpable tumour, on day 30 post injections the second mouse had a palpable tumour and the third mouse showed no sign of tumour development.

Mice were continued to be monitored, measuring mice body weights and tumour sizes. When the tumour from the first mouse reached about 10mm diameter, the mouse was humanely euthanized in CO2 and the tumour harvested. A single cell suspension was prepared from the tumour, cell viability and total cell count performed using trypan blue exclusion. Cells were then cultured and expanded in 1x25T and then 1x 75T flasks. After 24 h, cells were washed with PBS 1x to remove dead cells and debris. By day 4, the cells had reached 70 % confluency and 2 vials were frozen down for future use and the other cells were cultured for further injections. NeuTL cells prepared from the harvested

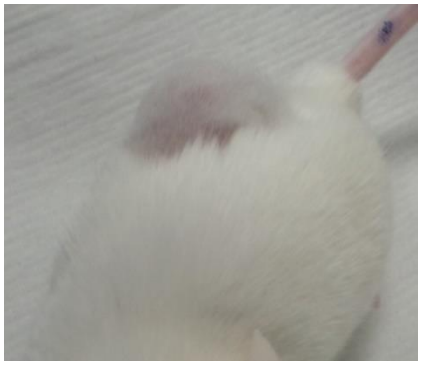
tumour were noted to no longer lift from the bottom of the flasks upon reaching 70 % confluency.

NeuTL cell injection trial 3

Cells from the harvested tumour were grown to 60-70 % confluency, injected into a group of 3 mice at a concentration of $2x10^6$ cells in 200 µL PBS 1x whilst maintaining the same preparation and treatment conditions. On day 7 post injections all 3 mice had palpable tumours. On day 14 post injection, tumours had reached an average diameter of about 10mm (about same size as the tumour on mouse 2 from *Cell injection trial 2*).



FVB/N spontaneous tumour day 30 post initial appearance



FVB/N subcutaneous tumour day 14 post cell injection

Figure 5: FVB/N c-neu mice spontaneous breast tumour vs implanted subcutaneous tumour

Tumours implanted subcutaneously grew at a faster rate than tumours in cell injection trial 2, see Figure 5 above. Cell injections were decreased to $1x10^6$ in 100 µL PBS 1x to slow the size of the down tumour growth (doubling size), to ensure that tumours did not grow above the AEC approved maximum diameter of 20 mm.

Drug Injection

FVB/N c-neu mice around the age of 8-10.5 months with spontaneous tumours (250-350 mm^3) or alternatively, FVB/N c-neu mice implanted with subcutaneous $1x10^6$ NeuTL murine breast cancer cells, on top of the right back flanks were used for the experiments. MAC treatment was administered intratumorally by directly injecting into the centres of the visible/palpable tumours on female mice. 50 µL treatment doses (vehicle TPGS and 4 % v/v MAC) were administered by using a 1 mL graduated syringe with a 30 gauge needle every 3 days over a period totalling 12 days, monitoring mice every day to check for general health and other physical discomfort. The health of mice was monitored using a health score sheet (example Table 10 below). Should tumours reach end point measurements or if mice showed a significant deterioration of health (inactivity, hunching, ruffled fur etc.), mice were humanely sacrificed using CO_2 asphyxiation followed by cervical dislocation and tumours harvested.

Table 10: Example of the health score sheet used to rate mice health

Signs	**0**	**1**	**2**	**3**
Activity	normal	isolated, abnormal posture	inactive or overactive	moribund or fitting
Alertness	normal	dull or depressed	little response to handling	unconscious
Body condition	normal	thin	loss fat, failure to grow	loss of muscle
Body weight	normal	reduced growth	chronic weight loss >15 %	chronic weight loss >20 %
Breathing	normal	rapid , shallow	rapid, abnormal	laboured, irregular, skin blue
Dehydration	normal	skin less elastic	skin tenting	
Eyes	normal	wetness or dullness	discharge	eyelids matted
Movement	normal	slight uncoordination	uncoordinated	Staggering , limb dragging, paralysis
Nose	normal	wetness	discharge	coagulated
Urine	normal		Abnormal colour or volume	No urine in 24 h or soiled perineum
Vocalization	normal	squeaks when palpated	struggles and squeaks loudly when handled	abnormal vocalisation

Score judgement: 0-4 = Mild, 5-8 = Moderate, 9-12 = Substantive.

Accumulative score of 5 or more: close monitoring required, accumulative score of 8 or more: humane endpoint, any score of 3 detected: seek immediate attention

Measurement of tumour size

Manual callipers were used to measure tumour dimensions (length and width) before treatment and every 3 days during treatment period. The formula; *Tumour Volume = L x W^2 x 0.52* was used to measure tumour size and treatment was initiated when tumours reached the size of approximately 300-500 mm^3.

Euthanasia of mice

Mice were humanely euthanized under CO_2 followed by cervical dislocation at the endpoint of each experiment. Mice were also humanely euthanized if tumours had reached diameter measurements greater than 20 mm or if mice had suffered significant health deterioration such as significant weight loss, severe lack of self-grooming, laboured breath and hunching back.

Harvesting of tumours

All procedures were performed under sterile conditions in a biological safety cabinet. Humanely euthanized mice were sprayed until soaked with 70 % ethanol, before being transferred into the biological safety cabinet. A small incision was made in the skin at the top of the tumours using a sterile surgical scissors. Skin was pulled back using sterile forceps exposing the tumour. Tissue around the tumour was gently excised freeing the tumours. Harvested solid tumours were weighed in a culture dish, transferred into labelled 6 well plates containing DMEM media supplemented with 10 % FBS and 1 % penicillin/streptomycin. The plates containing samples were stored on ice until ready for further processing.

Preparation of immune cells from murine tissues

All single cell preparations were done on ice under sterile conditions in a Biological Safety cabinet. Cell suspensions were prepared by dissecting the isolated tumours into small pieces using sterile surgical scissors, treating with serum-free DMEM/70 U/mL collagenase (Sigma) then mechanically dissociating cells by passing

through a 70 µm nylon sieve (Becton Dickson). Cells were washed twice with the staining buffer (2 % FBS + PBS 1x), centrifuged at 1500 rpm for 5min. After discarding the supernatant, collected cell pellets were re-suspended in staining buffer and stored on ice ready for further analyses. Viability of cells was assessed by staining cells with trypan blue exclusion dye, counted using a hemocytometer and a light microscope. Analysis and staining of the isolated tumour infiltrating neutrophils was performed following a series of treatment protocols [74, 75]. Continuous density centrifuge gradient was used to obtain enriched populations of tumour-infiltrating neutrophils for analysis of the neutrophil nuclear morphology and determination of the cytotoxicity of neutrophils.

Flow cytometry

Flow cytometry is commonly used to detect cellular physiology, gene expression, cell morphology, cell surface and intracellular protein expression in heterogeneous cell suspension mixtures at a very high speed [116]. To analyse immune cell populations, flow cytometric data was acquired using the Fortessa LSR II flow cytometer and analysed using the Diva and Flow Jo software. Instrument settings and parameters was set using the following controls; unstained cells, single fluorophore stains, fluorescence minus one (FMO) and single stains of ultra-comp beads to set the compensation parameters.

Fluorophore conjugated monoclonal antibody titrations to determine optimal dilutions for immunostaining of cells.

To reduce back ground staining and to determine optimum staining concentrations for each monoclonal antibody (mAb), antibody titrations were first carried out. Serial dilutions of conjugated antibodies were prepared using staining buffer (2 % FBS/PBS 1x) in clear bottom 96 well plates using different antibody amounts from 2 µL- 0.125 µL (1/25-1/400). Preparations of single cells in suspension were first stained with a fixable viability dye (1 µL in 1 mL PBS 1x), incubated at 4 ^{o}C for 20 min and then washed with the staining buffer. Resuspended cells were diluted to a concentration of $1x10^{6}$ in 50 µL staining buffer. 50 µL of cell suspensions were transferred to a separate 96 well U bottom plate before staining with the corresponding antibody concentrations, with an unstained sample also included as control. For immunostaining, the cells were centrifuged at 1250

rpm for 5 min and the supernatants discarded and then the cell pellets were resuspended with 50 µL of the corresponding fluorophore conjugated antibody concentrations using a multi-channel pipette and incubated at 4 ^{o}C for 30 min. After incubation and immunostaining, the cells were washed twice with the staining buffer and fixed in 2 % formaldehyde for 15 min. Cells were washed, resuspended in staining buffer and transferred to flow cytometry tubes (350 µL-500 µL). For longer storage, the immunostained cells were resuspended in 1 % formaldehyde and stored at 4 ^{o}C before flow cytometry analysis. Cells were analysed using the BD Fortesa II (FACS Diva software) flow cytometer. Results are shown below in Figure 6 below.

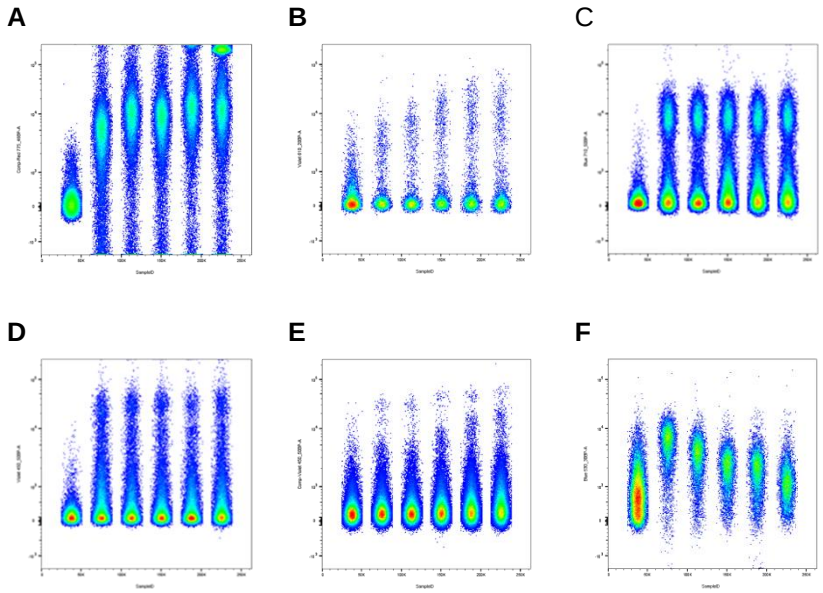


Figure 6: Fluorophore conjugated monoclonal antibody titrations and flow cytometry results

Results of samples stained with serial dilutions of conjugated monoclonal antibodies from 2 µL - 0.125 µL) (A) Dead/live Fixable dye_eFluor 780 (B) CD45_Super Bright 600 (clone 30-F11), (C) CD11b-PerCPCy5.5 (clone M1/70), (D) Ly6G_Horizon V450 (clone 1A8), (E) Ly6G_Pacific Blue (clone 1A8), (f) C-Met_FITC (eBioclone 7)

The Stain Index (SI) for each antibody was calculated using Equation 3.

$$SI = MFI_1\text{-}MFI_2) \div (2 \times SD)$$

Where, MFI_1= Mean Fluorescence Intensity of positive population
MFI_2 = Mean Fluorescence Intensity of negative population
SD = Standard deviation

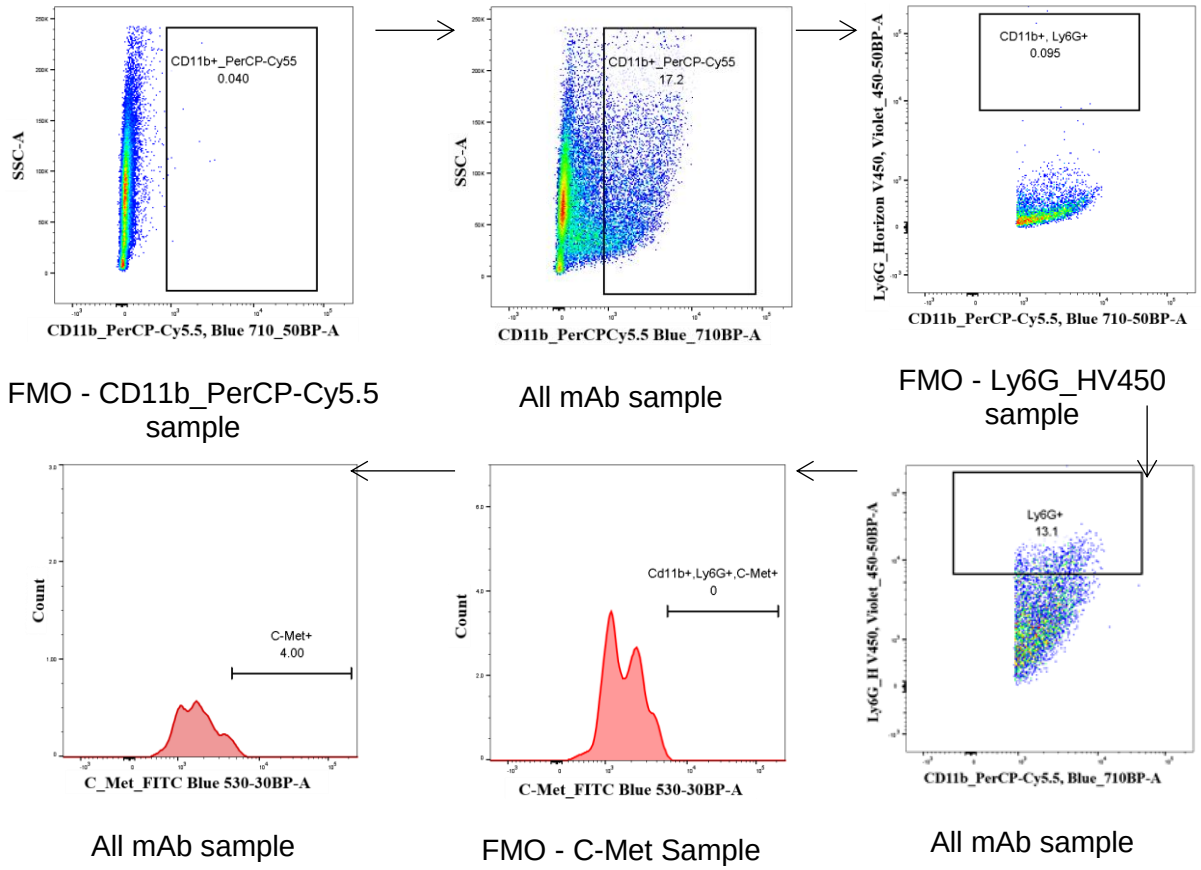
0.5 µL (1/100) of antibody concentrations in 50 µL of 1 x 10^6 cell suspensions were determined as optimum staining concentrations for Dead/live Fixable dye eFluor 780, CD11b_PerCP-Cy5.5, C-Met_FITC and CD45_Super Bright 600. For Ly6G_Pacific Blue 1 µL (1/50) in 50 µL of 1 x10^6 cell suspension was used.

Cell surface staining

Single cell suspensions prepared from tumours were stained for cell surface antigen markers (Live/dead, CD45, CD11b, Ly-6G (1A8), C-Met) following the previously reported cell surface staining protocol guidelines [74]. Each group analysis contained cell

samples with an unstained sample and fluorescence minus one (FMO) cell samples as negative controls, single stain samples as positive controls and a sample stained with all antibodies simultaneously. Ultra-Compensation beads were single stained with each antibody fluorophore and prepared under the same conditions as for the cells. To avoid nonspecific binding of antibody to Fc receptors, cells were incubated in 1 % BSA at 4 ^{o}C for 15 min. Cells were first stained with 1 µL fixable viability dye in 1 mL PBS 1x and incubated for 20 min at -4 ^{o}C in the dark. After incubation, cells were washed with 4 mL 2 % FBS/PBS 1x (staining buffer), followed by centrifugation at 1500rpm for 5 min and resuspension at a concentration of $1x10^{6}$ - $5\ x10^{6}$ cells in 50 µL staining buffer. Cells were stained in U bottom shaped 96 well plates with 1-2 µL of applicable fluorophore-conjugated antibodies (Table 8), followed by incubation at -4^{o}C in the dark. Cells were washed again twice with 200 µL staining buffer, fixed with 2 % formaldehyde and stored at 4 ^{o}C in 1 % formaldehyde/staining buffer ready flow cytometry analysis. Murine neutrophils were defined as $Ly6G^{hi}CD11b^{+}$ and C-MET expression was analysed as a specific marker for cytotoxic tumour infiltrating neutrophils.

Neutrophil gating strategy using controls



FMO - CD11b_PerCP-Cy5.5 sample

All mAb sample

FMO - Ly6G_HV450 sample

All mAb sample

FMO - C-Met Sample

All mAb sample

Figure 7: Flow cytometry controls used for establishing the flow cytometry gating strategy

First, compensation beads stained with single fluorophores for each antibody were used for compensation of fluorophores to calculate and correct for spectral overlap. Unstained samples and Fluorescence minus one (FMO) samples were used as negative controls and single fluorophore samples as positive controls to set the flow cytometric gates for neutrophils.

Neutrophil identification gating strategy

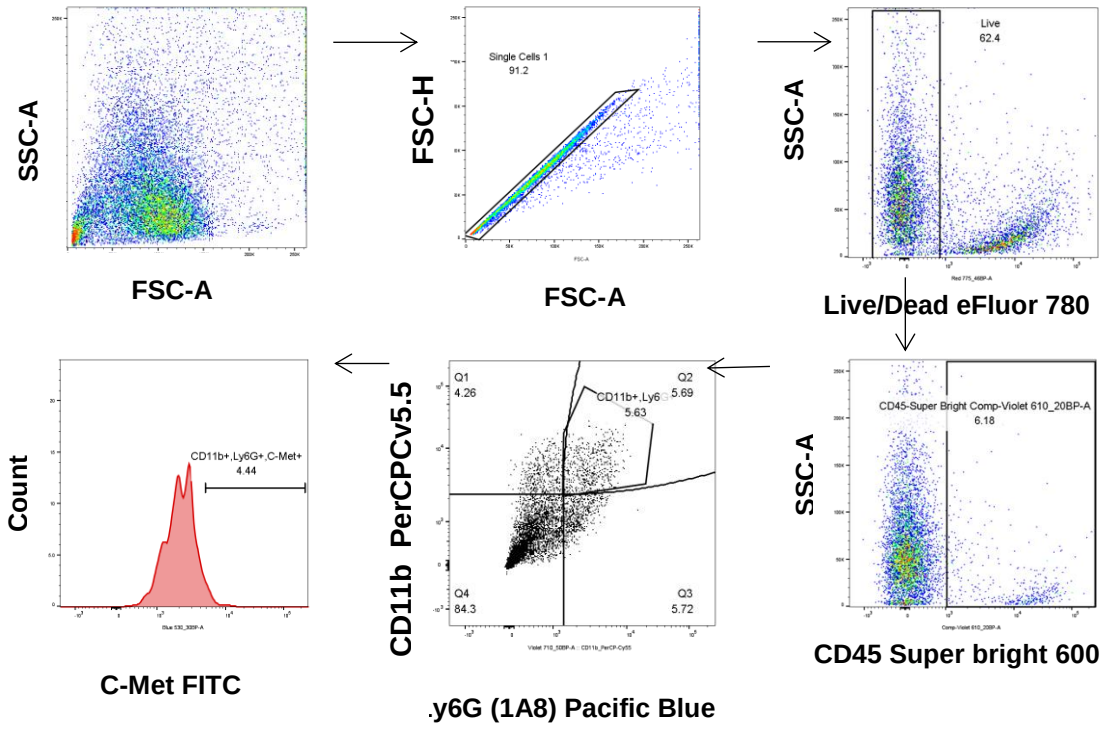


Figure 8: Flow cytometric gating strategy analyzing for tumour associated neutrophils
Single cells were first identified by cleaning cells, removing debris and doublets through a series of gates P1-P4 (FSC-A vs SCC-A, FSH vs FSC-A, SSW vs SSH and FSW vs FSH respectively. P3 and P4 Gates were excluded from the illustration above). Live cells were identified using the Live/Dead fixable viability dye eFluor 780 and the negative population was identified as the live cells. The $CD45^+$ gate was used to identify the leukocyte population from the live cells. The neutrophil population was identified by plotting the specific murine neutrophil marker Ly6G_Horizon v450 (1A8) against CD11b_PerCP-Cy5.5, with the positive population indicated in Quadrant 2. $Ly6G^+$ $CD11b^+$ cells were then plotted against the c-Met_FITC to determine levels of c-Met expressing neutrophils.

Isolation of neutrophils from tumour tissue

Continuous gradient separation of neutrophils

After cell counting, cells were washed and resuspended in 3mLs of ice chilled staining buffer (2 % FBS/PBS 1x). Histopaque density 1.119 and density 1.077 were brought to room temperature before use. 3mLs of prepared cell suspensions were carefully layered on top of 3mLs Histopaque 1.119 and 3 mL Histopaque 1.077 respectively in 15 mL centrifuge tubes. Samples were centrifuged at 25 ^oC, 872 x g for 30

min with the brake off, using the HERAEUS MULTIFUGE X 1R centrifuge (Thermo Scientific). Isolated neutrophils were collected between the Histopaque 1119 and 1077 interface. Figure 9 shows captured images of cell suspensions before and after continuous gradient centrifugation. Purified neutrophils were washed twice with the staining buffer, centrifuged at 1500 rpm for 5 min at RT. Viability and count of purified neutrophils was assessed using the trypan blue exclusion and hemocytometer with a light microscope. Purified neutrophils for cytotoxicity determination were resuspended in DMEM media supplemented with 10 % FBS and 1 % penicillin/streptomycin and then further serially diluted (E: T ratio of 20:1down to -1.25 : 1 TAN Effector to Target Neu TL cancer cell ratios) as final effector cell density. For preparation of slides and microscopy analysis, the purified TAN cells were resuspended at 1 x 10^5 cells in 50 µL staining buffer (2 % FBS/PBS 1x).

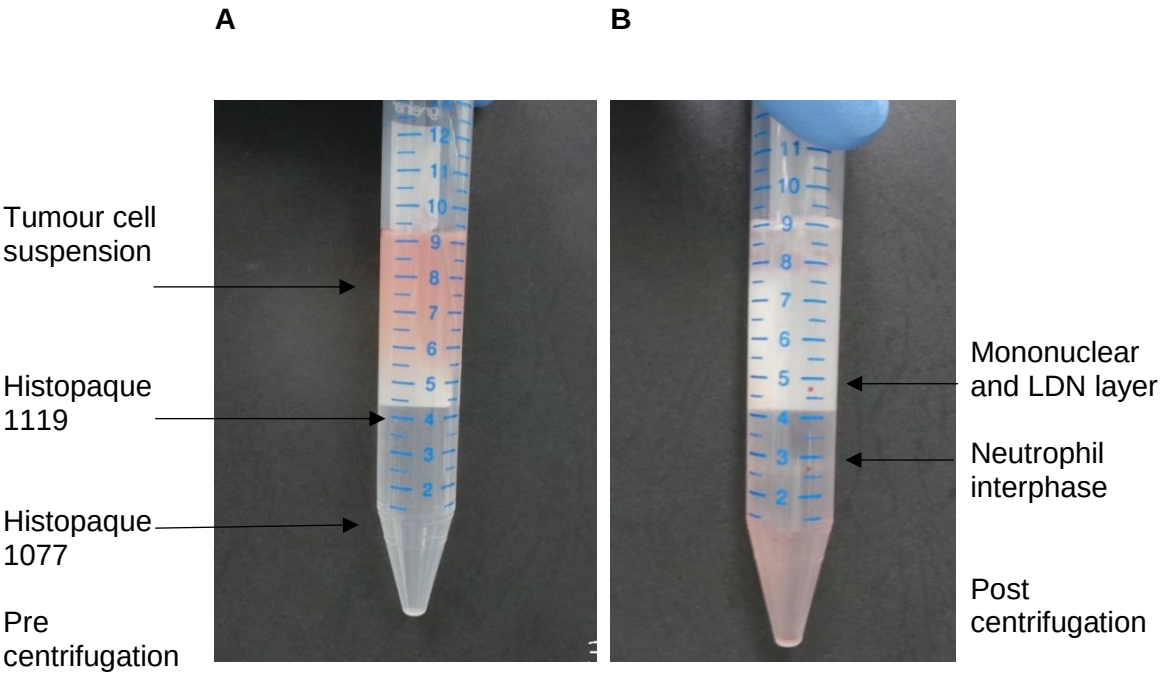


Figure 9: Purification of neutrophils using continuous gradient separation (Histopaque gradient 1.119 and gradient 1.077) from tumour cells

(A) Image of a 15 mL centrifuge tube containing layers of 3 mL histopaque gradient 1.119, 3 mL histopaque gradient 1.077 and 3 mL tumour cell suspension before centrifugation (B) Image of a 15 mL centrifuge tube taken after continuous gradient separation with visible mononuclear (LDN) and neutrophil (HDN) layers.

Immunocytochemistry

Cytological staining of neutrophils

Preparation of cell suspension on slides for microscopy

Slides of cell smears were prepared following a series of protocol guidelines [75]. Neutrophils collected from the Histopaque 1119 and 1077 interface were washed twice with the staining buffer and centrifuged at 1500 rpm for 5 min. Cells were resuspended at 1 x10^5 cells in 50 µL and gently smeared across Superfrost^R plus slides (Thermo Scientific). Glass slides were left to air dry for 5minutes, fixed in 70 % ethanol for 2 min ready for staining. Slides were first dipped 5-6 times in distilled water, stained in Methylene blue solution for 2 min or Haematoxylin solution for 3 min, washed under tap water for 1 min, then counter stained with Eosin Y solution for 10 sec. Slides were washed again under tap water for 1 min, dehydrated by rinsing the slides in increasing ethanol concentrations (70 %, 96 % and 100 %). Slides were allowed to air dry, mounted with DEPEX mounting media then covered with a cover slip. Nuclear morphology was assessed using the OLYMPUS BX53 using cellsSens Entry software.

Cytotoxicity Assays for neutrophil killing of cancer cell targets

Preparation of SYTOX Green nucleic acid stain working solution

SYTOX Green nucleic acid stain was acquired from ThermoFisher Scientific as 5mM solution in DMSO. Small aliquots of the solution were kept at -20 ^oC, protected from light and thawed before use. 4 µM SYTOX Green nucleic acid stain working solutions were prepared by diluting 4 µL of the stock solution (5mM SYTOX Green) with 5 mL of warm DMEM phenol red free media. 50 µL of the prepared working solutions were added to the 96 well plates containing cells to a final concentration of 1 µM per well.

Neutrophil cytotoxicity and determination

For cytotoxicity assays, each experiment included n=3 replicates for each dilution of neutrophils as immune effector cells, either alone or added on top of the breast cancer cells as targets. NeuTL cells as target cell populations were pre-seeded into Black clear bottom 96 well plates at 4000 cells per well in 100 µL DMEM phenol red free media,

incubated at 3 ^{o}C, 5 % CO_2 for 24 h before cytotoxicity assay. Neutrophils were diluted across the 96 well microtiter cell culture plate, each well containing a lawn of cancer cells established the previous day in each well as targets or alone to monitor the cancer cell viability. The dilutions of added neutrophils were at Effector: Target ratios from 20:1 down to 0.063:1, to determine their potency for killing murine breast cancer cells (NeuTL cells) when co-incubated in combined culture. SYTOX green nucleic acid stain was added to the wells containing cells to a final concentration of 1 µM, for monitoring induction and activation of cell death over 4-6 h after addition of the neutrophils to the culture of cancer cells. Plates with rows of cells alone (neutrophils alone and cancer cells alone as controls) or combined together (neutrophils plus cancer cells) were incubated at 37 ^{o}C, 5 % CO_2. Cell death was assessed by measuring Sytox green nuclear fluorescent dye uptake (indicating death) into the cells at the start and over the duration of the assay using the BMG FluorSTAR OPTIMA plate reader. Cells were monitored by eye on the FITC channel using the OLYMPUS IX53 fluorescent microscope and cellSens Standard software.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B				20:1	10:1	5:1	2.5:1	1.25:1	0.63:1			
C												
D												
E												
F												
G												
H												

100 % Dead NeuTL cells control – 100 µL cells, 50 µL NP-9 solution, 50 µL SYTOX Green solution

100 % Live NeuTL cells control – 100 µL cells, 50 µL media, 50 uL SYTOX Green solution

Neutrophil Dilutions – 100 µL NeuTL cells, 50 µL Neutrophil suspension, 50 µL SYTOX Green solution

Neutrophil dilutions alone – 50 µL Neutrophils, 100 µL media, 50 uL SYTOX Green solution

Media alone – 150 µL media, 50 µL SYTOX Green solution

PBS 1x

Empty

Figure 10: Format for Neutrophil cytotoxicity and determination in 96 well plate set up

Determination of cell death

In vitro determination of cell death was performed using the fluorescent nucleic acid stain SYTOX Green (excluded from live cells); with the emission and excitation

values set at 485-P and 520-10 respectively using a BMG FLUOstar OPTIMA plate reader (BMG LABTECH Australia).

$$\%\ Specific\ killing = \frac{[(NeuTL\ murine\ cancer\ cells + Neutrophils) - (Neutrophils\ alone) - (100\ \%\ Live\ cancer\ cells)\ ]}{(100\ \%\ Dead\ cancer\ cells - 100\ \%\ Live\ cancer\ cells)} x100$$

Statistical analysis

Each experimental study was repeated two to three times to test for reproducibility of the data. For orthotopic transplanted tumour models, data was analysed by one-way analysis of variance (ANOVA) with Fisher's least significant difference method for pair wise comparisons. For all other experiments, n=3 replicates per assay point was used and the Students T test applied to determine levels of error and significance of the data, with $p<0.05$ considered significant. GraphPad Prism v 8.0 software was used for analyses, unless otherwise indicated.

Chapter 4: Results

Anticancer and immunomodulatory activity of MAC in pre-clinical murine breast cancer model

Injection of 4 % MAC intratumourally impaired tumour growths of transgenic FVB/N c-neu spontaneous tumours.

Several studies have reported the antitumor activity of TTO and its main bioactive compound, terpinen-4-ol, against various cancer cell lines *in vitro* and in murine models [12, 15, 16, 26, 107, 111, 117]. The effect of different doses of MAC (neat or undiluted, 10 %, 4 % and 1 %, diluted in 0.5 % TPGS) on spontaneous tumours of the female transgenic FVB/N c-neu murine breast cancer model were previously investigated in the Ralph laboratory by a PhD student [14]. Injecting 1 % MAC and 4 % MAC into solid tumours of mice over a period of 30 days were shown to cause more than 2-fold and 6-fold less tumour volumes in comparison to controls, respectively. Injecting 4 % v/v MAC directly into solid tumours of FVB/N c-neu mice was found to induce tumour cell death without causing any observed toxicity signs. Hence, the 4 % v/v MAC dose was chosen as suitable, safe and effective for the present study.

Mice around the age of 8-10.5 months with spontaneous or implanted tumours were used for the experiments in the present study. Mice bearing tumours (250-350mm^3) were placed in 3 groups of 7 (N=21, n=7) and received treatment every 3 days for 12 days (5 x 50 µL injections). The first group was injected intratumourally with 4 % v/v MAC, the second group with 0.5 % TPGS as vehicle control, and the third group received no treatment. Results from the present study were consistent with findings from the previous study, as injecting 4 % v/v MAC directly into FVB/N c-neu mice solid tumours lead to shrinkage of tumours. Tumours treated with 4 % v/v MAC had significantly lower average tumour volumes (327.48 mm^3 ± 11.77, $p<0.0001$), as compared to tumours injected with 0.5 % TPGS (693.98 mm^3 ± 36.9) and untreated control tumours (656.75 mm^3 ±45.9) as presented in Table 11. At endpoint, weights of harvested tumours were recorded and tumours harvested from mice treated with 4 % v/v MAV weighed significantly less (0.97g ± 0.06, $p<0.001$), when compared to 0.5 % TPGs vehicle control (1.56g ± 0.10) and

untreated control (1.60g ± 0.09) tumours. However, there were no significant differences in mean tumour volumes and mean tumour weights between vehicle and control groups, with p values >0.05.

To monitor general health and toxicity of treatment, a health score sheet (example in Table 9) was used to rate the mice using clinical signs (physical discomfort, breathing, dehydration, movement, urine, vocalisation, hunching and grooming). Mice treated with 4 % MAC showed no signs of toxicity (scored normal or accumulative score <2) throughout the experiments and were observed to be active and well-groomed, the same as the control mice to day13 (endpoint). In addition, a comparison of the average relative body weights among treatment groups showed no significant differences, $p > 0.05$. The minor changes that may have been observed in relative body weights of treated mice were attributed to the reduction of tumour sizes.

The results indicated that 4 % v/v MAC was able to kill tumour cells slowing down tumour growth, and consequently reducing tumour weights of treated mice in comparison to untreated tumours or vehicle only treated tumours. The results were consistent with previous studies at Griffith University in the Ralph laboratory, where 4 % v/v MAC was reported to inhibit growth of spontaneous tumours in transgenic FVB/N c-neu mice and syngeneic 4T-1 subcutaneous tumours in Balb/c murine models, with no observed signs of adverse side effects from the treatment in both murine breast cancer modes [14]. The results are presented in Table 11 and Figure 11 below, respectively.

Table 11: Comparison of FVB/N c-neu mice mean relative body weights, tumour volumes and weights post treatment

Treatment	No. of mice	Ave body Weight (g)	Tumour Vol. ($mm^{3)}$	Tumour Weight (g)
Control	7	31.32(1.04)	656.75(45.9)	1.60(0.09)
0.5 % TPGS	7	30.67(1.08)	693.98(36.9)	1.56(0.10)
4 % v/v MAC	7	30.33(0.51)	327.48(11.77)****	0.97(0.06)****
			P values	
Control vs Vehicle		>0.05	>0.05	>0.05
Control vs 4 % v/v MAC		>0.05	<0.0001	0.0001
Vehicle vs 4 % v/v MAC		>.0.05	<0.0001	0.0002

Spontaneous and implanted FVB/N c-neu mice solid tumours (between 250-350 mm^3) were intratumourally injected every 3 days for 12 days, with 50 µL of 4 % v/v MAC, 0.5 % TPGS and the other group received no treatment. Relative mice body weights and volumes were recorded throughout the treatment. Harvested tumours were weighed at endpoint. Data was analysed using GraphPad Prism v 8.2 and one way ANOVA was used to evaluate the significance and comparison of group means. The values are represented as mean ± Standard Error of Mean (SEM) and p values reported. P-values: **** = < 0.0001.

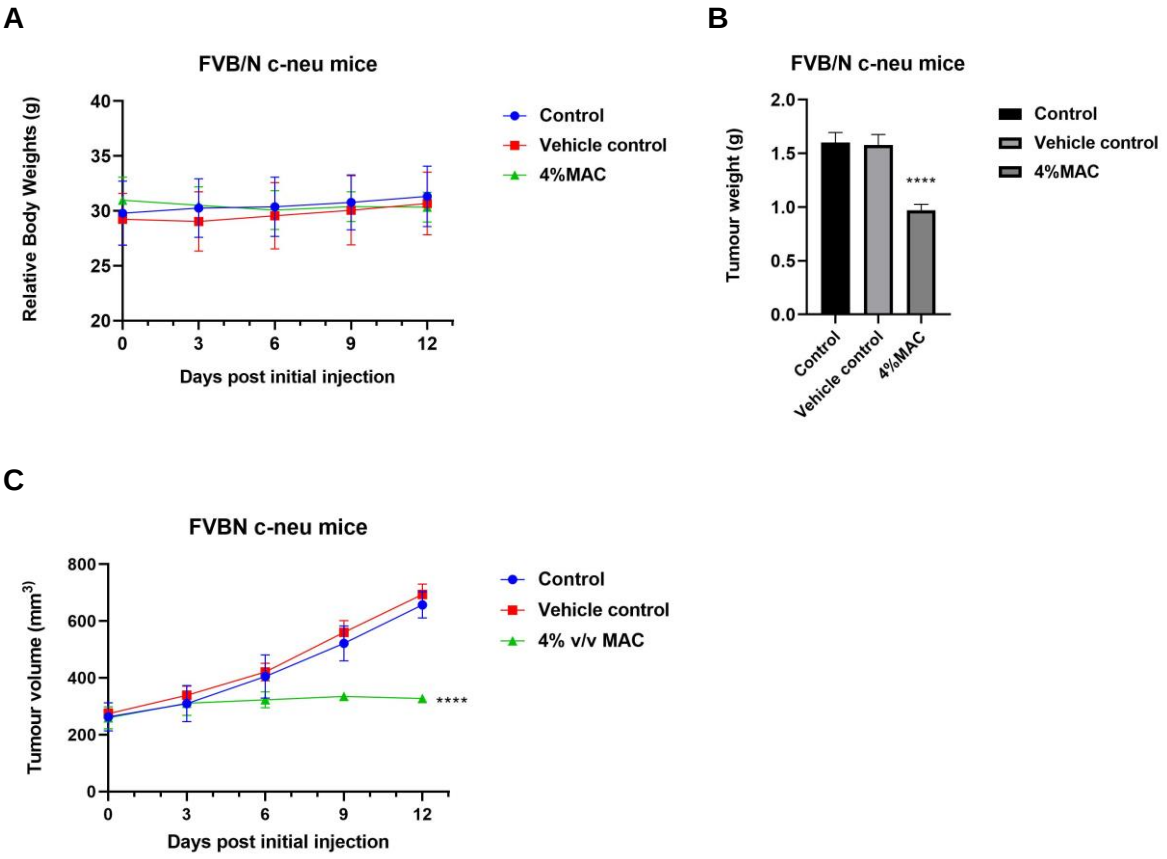


Figure 11: Characteristic representation of FVB/N c-neu tumours at day 13 post treatment Spontaneous and implanted FVB/N c-neu mice solid tumours (between 250-350 mm^3) were intratumourally injected every 3 days for 12 days, with 50 µL of 4 % v/v MAC, 0.5 % TPGS and the control group received no treatment. Relative mice body weights and volumes were recorded throughout the treatment. Harvested tumours weights were recorded at endpoint. For plots, n=3 replicates per assay point was used (A) Relative mice body weights (B) Average tumour weights (g) on day 13 (C) Average tumour volumes. Data was analysed using GraphPad Prism v 8.2. Results expressed as the mean ± SEM. Error bars represent ± SEM. P-values: **** = < 0.0001.

Characteristics of FVB/N c-neu murine breast cancer model tumours post treatment

In recent years, the tumour microenvironment has increasingly become a target for studies aimed at investigating the immune modulating effects of therapy drugs. In previous studies, direct injection of therapy drugs into solid tumours has been mainly used to manipulate immune responses in solid tumours which aimed at investigating cytokine modifications at tumour sites (discussed in the review) [20]. The anticancer activity and immunological responses activated by MAC using female transgenic FVB/N c-neu spontaneous murine breast cancer model were investigated. The study aimed at investigating the short term effects of directly injecting 4 % MAC into solid subcutaneous tumours I Treatment was administered by directly injecting 50 µL of 4 % v/v MAC and 50 µL of 0.5 % TPGS as vehicle control every 3 days for 12 days. On day 13, all mice were humanely euthanized by CO_2 asphyxiation followed by cervical dislocation.

Photographic images of FVB/N c-neu mice tumours were taken before and after harvesting of tumours (Figure 12 below). The experiment was designed to allow harvesting of intact solid tumours at endpoint, in order to investigate the immunological responses (neutrophil infiltration in the tumour microenvironment) activated by directly injecting 4 % v/v MAC into the middle of solid tumours. Tumours harvested from mice treated with 4 % v/v MAC were observed to be more sanguineous compared to tumours harvested from the vehicle control (0.5 % TPGS) and untreated control groups. A void area surrounded by necrotic tissue was observed in the middle of tumours harvested from treated mice. This suggests an infiltration of blood into the drug treated tumours. Tumours treated with 0.5 % w/v TPGS had similar appearance as untreated tumours, which demonstrated that at 0.5 % TPGS was a safe emulsifier and did not induce antitumor cytotoxicity. TPGS is an FDA approved water soluble vitamin E nutritional supplement commonly used as a drug delivery vehicle [113].

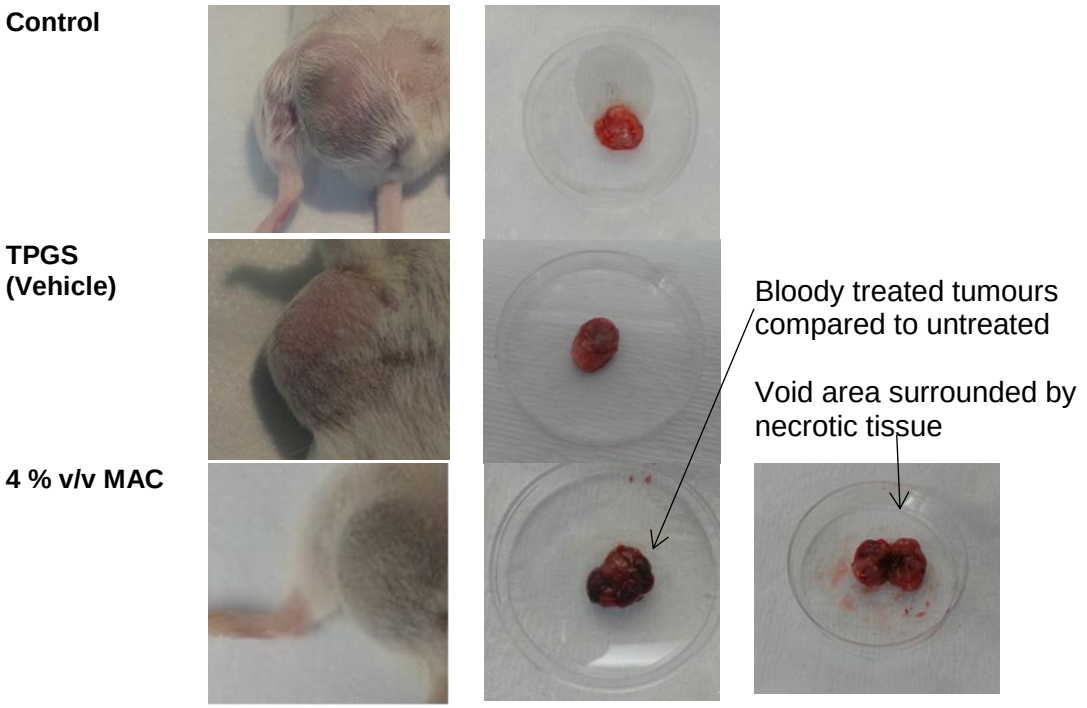


Figure 12: Transgenic FVB/N c-neu spontaneous murine breast cancer model images at day 13 post treatment

Spontaneous FVB/N c-neu mice solid tumours (between 250-350mm^3) were intratumourally injected every 3 days for 12 days, with 50 µL of 4 % v/v MAC, 50 µL 0.5 % TPGS and the control group received no treatment. On day 13, mice were humanely euthanized in CO_2 and solid tumours harvested. Photographic images of FVB/N c-neu mice tumours were taken. Tumours harvested from FVB/N c-neu mice injected with 4 % v/v MAC were observed to be sanguineous compared to tumours from the untreated control and vehicle control groups. Tumours harvested from mice injected with 4 % MAC were also observed to have a void surrounded by necrotic tissue in the middle at the point of direct MAC injection deposits.

Flow cytometry

Identification of tumour infiltrating neutrophils

Neutrophils are primary responders during inflammatory conditions and around damaged tissue to fight against invading pathogens. Mobilized neutrophils become activated upon reaching target tissue and use a range of mechanisms to mediate an even more expanded immune response [76]. To determine the population of tumour infiltrating neutrophils, single cell suspensions were prepared from tumours harvested at the end of each treatment. Cell samples were stained with a range of fluorophore conjugated mAbs to define the cell types. Murine TANs were identified as $CD11b^+$ $Ly6G^+$ and c-Met expression on neutrophils was also assessed.

To identify murine neutrophils the gating strategy previously described in Figure 7 and Figure 8 were used. Following the removal of doublets, live cells were identified through the live/dead gate and neutrophils were identified through the $CD11b^+Ly6G^+$ gate. In the present study tumours treated with 4 % v/v MAC had higher levels of neutrophil counts (5.33 %) compared to the 0.5 % w/v TPGS (2.89 %) and control groups (2.24 %) (Results are presented in Figure 13 below). The results also showed that at 0.5 % w/v TPGS did not activate any observed immunological responses, as the results were similar to untreated tumours.

C-MET expression on tumour infiltrating neutrophils has been highly associated with neutrophil transendothelial migration and tumour cytotoxicity in a recent study [17]. Furthermore, the expression of c-MET on $CD11b^+Ly6G^+$ cells was investigated. $CD11b^+$ $Ly6G^+$ neutrophil cells were further gated through the c-MET^+ gate to identify $CD11b^+Ly6G^+$ expressing c-MET. The results showed that compared to neutrophils from vehicle (2.89 %) and control (2.24 %) tumours, neutrophils from tumours treated with 4 % v/v MAC (3.74 %) had higher levels of c-MET expression, which correlated with the higher TAN counts. C-MET is expressed on some cancer cells and cytotoxic neutrophils in cancers hence, it was not surprising to identify neutrophils that lowly expressed c-MET

from vehicle and control tumours. Therefore, the results indicated that c-Met expression on neutrophils was increased by treatment of tumours with MAC, meaning killing of cancer cells in the tumour microenvironment may actually cause infiltrations of tumours by antitumor cytotoxic immune cells. However, the mechanisms which lead to differentiation of neutrophils into the anticancer cytotoxic phenotype are yet to be fully understood. The question is whether neutrophils can be re-educated to recognise and kill cancer cells using different stimuli in the tumour micro environment or the ability to kill cancer cells is only limited to certain phenotypes.

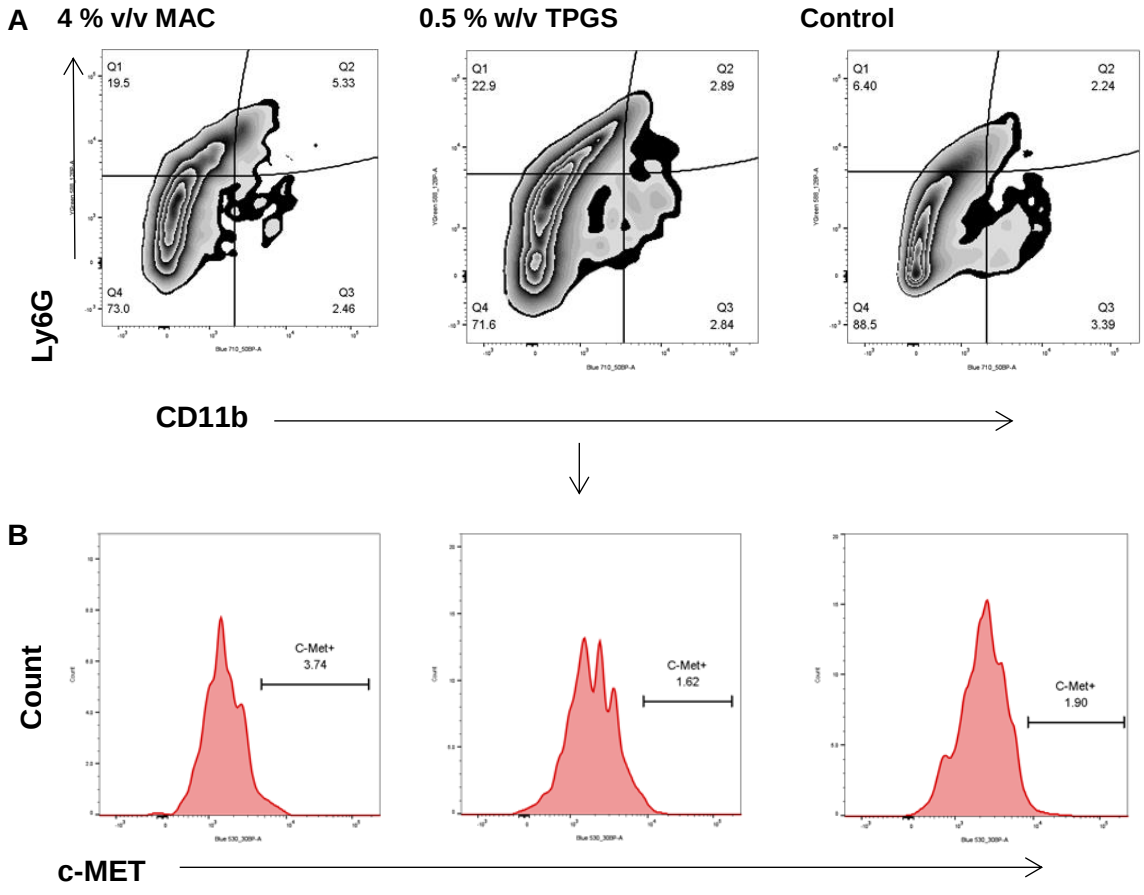


Figure 13: Population levels of neutrophils expressing c-MET analysed by flow cytometry (from treated, vehicle and control tumours)

(A) Identification of murine neutrophils using Density plots in quadrants (CD11b_PerCpCy5.5) vs Ly6G Horizon V450 (B) Identification of c-MET expressing neutrophils using Histograms ($CD11b^{+}Ly6G^{+}$ cells vs c-MET_FITC). Cells were first gated through a series of gates to remove debris and doublets (using FSC-H vs FSC-A, FSW vs

FSCH, SSW vs SCH). Live cells were identified through the negative population on the FV Live/dead eFluor 780 gate (FV Live/dead eFluor 780 irreversibly stains dead cells). Live cells were then analysed by identifying neutrophils through the CD11b_PerCpCy5.5 (clone M/70) vs Ly6G_Pacific Blue/Horizon V450 (clone 1A8) double positive gate (Quadrant 2). In addition, $CD11b^{+}Ly6G^{+}$ cells were further analysed for c-MET expression through the c-MET_FITC (eBioclone 7) positive gate. (Supplementary Data in Appendix I).

During optimisation of methods, CD45_Super Bright 600 (clone 30-F11) was included in the gating strategy to identify the leukocytes positive population first. However, the antibody was too dull to clearly identify negative versus positive positions and therefore; it was removed from the gating strategy. Furthermore, the study was also aimed at investigating the population of lymphocytes including Tregs, but unfortunately the antibodies were also not working and the analysis was excluded.

Immunocytochemistry

TANs isolated from FVB/N c-neu murine tumours treated with 4 % v/v MAC exhibited a polymorph-nuclear diversity

Traditionally, neutrophils have been identified using basic nuclear morphological features, content of stain positive granules and size. However, recent studies have highlighted the heterogeneity of murine neutrophils and their controversial roles in cancer progression [6, 8, 72]. Neutrophils were isolated from treated tumours by continuous density gradient separation and cell slide smears were prepared from resuspended cell pellets (at 1 x10^5 cells in 50 µL staining buffer) using a series of protocol guidelines [75]. Cell slides were fixed and stained with either haematoxylin solution or methylene blue solution, then counterstained with eosin solution per standard protocol but optimised to achieve best staining result.

Stained slides were examined (neutrophil nuclear morphological characteristics) using the OLYMPUS BX53, cellSens Entry using the 100x oil immersion objective. The results showed a diverse neutrophil population with different nuclear morphological features (round, segmented and hypersegmented). To determine the estimate percentage of neutrophil subpopulation per field view (n=10), neutrophil subpopulations were manually counted from images captured from random fields at 100x oil immersion objective. Cell subpopulation percentages were calculated by dividing the average total count of each cell subpopulation by the average total count of cells per view, and then multiply by 100. The population of neutrophils with a hypersegmented nuclear morphology were the more dominant phenotype (~ 62.72 %). Immature neutrophils constituted ~5.88 %, mature neutrophils (lobular/segmented nucleus) ~9.8 % and other cell types constituted~21.56 %. In previous studies, immature neutrophils were found on the LDN layer; therefore the small proportion of immature neutrophils observed on the slides could have come from residual cells from the top mononuclear layer (contamination during analysis. Previous reviews on neutrophils in cancer have highlighted the diversity

of circulating neutrophils in cancer, although the sub-populations were usually identified from different organs such as bone marrow, spleen and peripheral blood [63, 118].

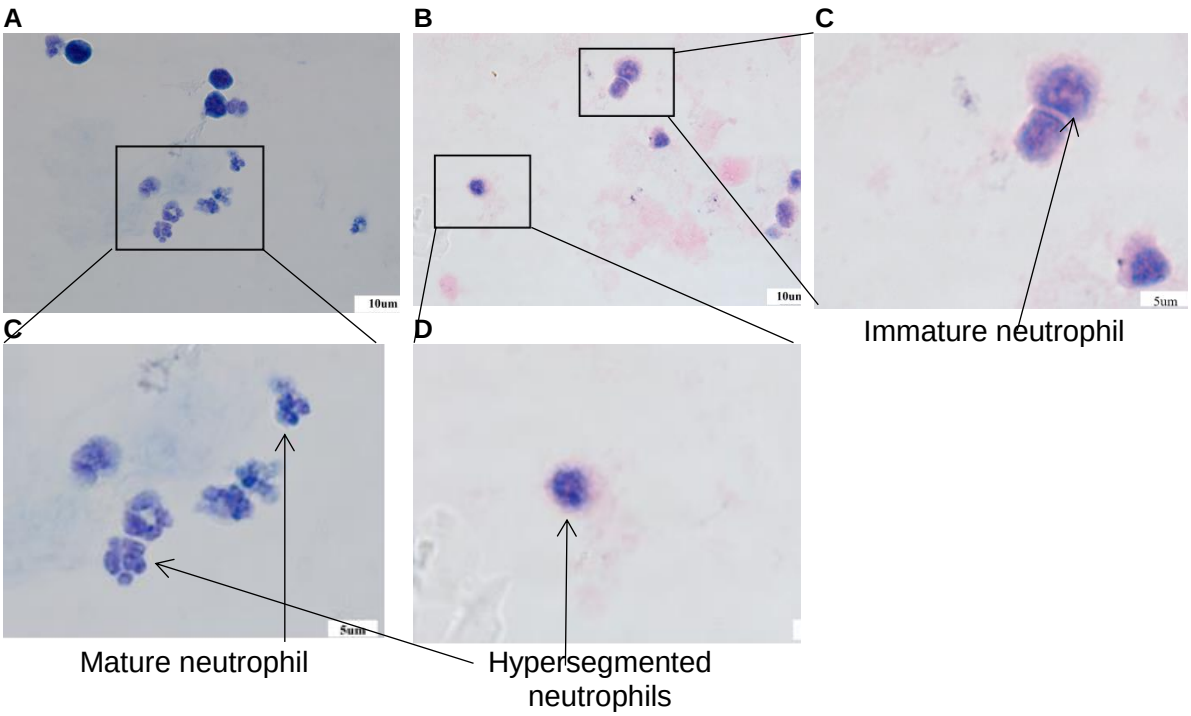


Figure 14: The morphology of TANs isolated from FVB/N c-neu tumours treated with 4 % v/v MAC

Neutrophils were isolated from tumour cell suspensions by discontinuous density gradient separation, fixed and stained with (A and C) Methylene blue solution or (B, C and D) Haematoxylin solution, then counter stained with Eosin Y solution. Slides were assessed using a light microscope at 100 x oil immersion objective (OLYMPUS BX53, cellSens Entry). (C and D) Zoomed in images (5 µm) of hypersegemented TANs and mature neutrophils (C) zoomed in image (5 µm) of an immature neutrophil.

Determination of neutrophil cytotoxicity

Purification of neutrophils by continuous gradient centrifugation

To determine the potential cytotoxicity of infiltrating neutrophils from tumours, prepared cell suspensions were isolated by continuous centrifuge gradient separation using Histopaque step gradient with 1.119 and 1.077 g/mL solutions. The purified neutrophils were collected and harvested from between the interface of the Histopaque solutions of density 1.119 and 1.077 g/mL. Viability of the isolated neutrophils from tumours was assessed using the trypan blue dye exclusion test. The results showed that the purified neutrophils were contaminated by a significant proportion of dead and live cells, with this co-isolated proportion constituting about ~15 % dead tumour cells, ~15 % live cells and ~70 % live neutrophils (trypan blue exclusion).

The purity of isolated neutrophils was also determined to be less than 60 % after further manipulation from the staining and after analysis of live/dead cells by flow cytometry. Viability of isolated neutrophils was also determined by assessing SYTOX Green uptake in 96 well plate wells which contained neutrophils alone at time 0 h and after layering neutrophils on top of NeuTL murine cancer cells in parallel wells. The wells were observed using a fluorescence microscope on the FITC channel. The results from the fluorescence analysis consistent with trypan blue exclusion results. Images were taken over the time 0-48 h (Figure 15 below). The population of cells collected from between the interface of the Histopaque step gradient 1.119 and 1.077 g/mL was also assessed by immunocytochemistry. Cells were stained with 0.1 % Methylene blue solution or Hematoxylin solution, and then counterstained with eosin solution. The results showed that some other cell types as well as immature neutrophils were also co-purifying with neutrophils. Also, it should be noted that the neutrophils when cultured alone were not viable for more than several hours. Although the neutrophils appeared very healthy (> 95 % live at 0 h), significant levels of SYTOX Green dye uptake was visible in the micrographs by 3 to 6 h, after being cultured.

Neutrophils prepared for cytotoxicity assays examined by fluorescence microscopy

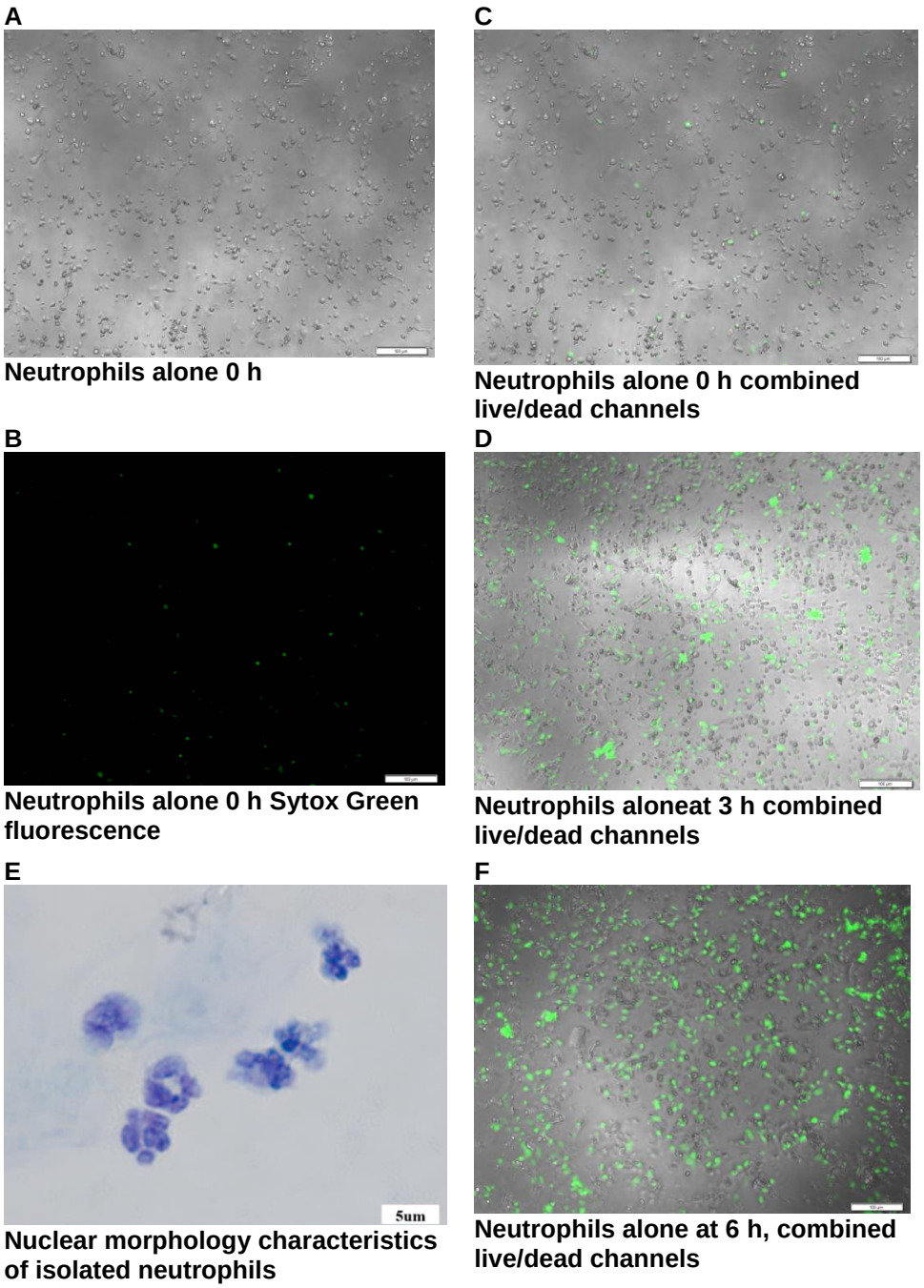


Figure 15: 96 well plate time–lapse images of purified neutrophils alone

(A) Neutrophils alone well image at 0 h, (B) fluorescence image of the neutrophils alone well at 0 h (C) overlayed images of combined channels to indicate viability of isolated neutrophils at the beginning of the neutrophil cytotoxicity assay (D) Neutrophils alone viability at 3 h (E) Nuclear morphology of isolated neutrophils (scale bar represents 5 µm) (F) Neutrophils alone viability at 6 h.

Viability of neutrophils ex vivo

At present, literature on the life span of neutrophils *in vivo* and *ex vivo* is filled with controversy and is yet to be well investigated. Previous studies have reported various estimated half-life spans of neutrophils *ex vivo*, although most studies aimed at manipulation of neutrophils in culture have been assessed within 24 h [86]. However, another study showed an estimated circulating half-life span of 12.5 h and 5.4 days for murine and human neutrophils respectively, using a mathematical model although the results were received with controversy [87, 88].

To monitor cell viability, continual fluorescence images were taken until 48 h. In the present study viability of neutrophils could be seen until 6 h, on fluorescence images captured from 96 well plates of the wells containing neutrophils alone. Furthermore, the cytotoxicity of neutrophils towards NeuTL murine cancer cells could be observed from images captured at 12 h and 24 h showing that neutrophils could have been still viable until then. Previous studies have suggested that different stimuli during inflammatory conditions prolong the life span of neutrophils [89]. Therefore, the life cycle of neutrophils *in vivo* and *ex vivo* is yet to be fully explained and understood.

Neutrophils isolated from transgenic FVB/N c-neu mice showed cytotoxicity towards the NeuTL murine cancer cells

In recent years, the antitumor cytotoxic activity of neutrophils has gained considerable attention and several murine and human studies have reported the anticancer immune mediating activity of neutrophils [5, 6, 17, 70, 79, 82, 119]. To determine the anticancer cytotoxicity of TANs, neutrophils were isolated from tumours treated with 4 % v/v MAC and evaluated for cytotoxicity. Neutrophils purified by continuous centrifuge gradient separation were washed and resuspended in warm phenol red free DMEM media. A dilution range with different ratios of neutrophils were layered above a constant number of NeuTL murine cancer cells (seeded the previous day) from effector-to-target ratios (E: T) of 10:1-1.25:1 (n=3) and then co-cultured, measuring SYTOX Green uptake by dead cells every 30minutes for 3-4 h, using the BMG LABTECH

FLUOstar plate reader. Wells of neutrophil cells alone and 100 % Live NeuTL murine cancer cells alone were also included.

Several assays have been used to measure the activity of immune cells *in vitro* and *in vivo* and in this study, the anticancer activity of neutrophils *ex vivo* was measured following previously used protocol guidelines [120]. The fluorescence intensity as a measure of killing of NeuTL murine cancer cells alone was measured and calculated using the following formula: % cytotoxicity = [(Fluorescence intensity of Neutrophils + NeuTL cancer cells) – (Fluorescence intensity of neutrophils alone) – (Fluorescence intensity of 100 % Live NeuTL cancer cells)] ÷ (Fluorescence intensity of 100 % Dead cancer cells – Fluorescence intensity of 100 % Live cancer cells). Time-lapse neutrophil cytotoxicity was calculated for each E: T cell ratio. GraphPad Prism v 8.2 software was used to analyse data from the plate reader and One way ANOVA was used for comparison of group mean values and p values reported. The results showed anticancer cytotoxicity by neutrophils isolated from tumours of mice treated with 4 % v/v MAC over the E: T (Figure 17). Neutrophil cytotoxicity towards NeuTL cells was significantly different across the E: T ratio range, $p < 0.0001$. Neutrophils at 10:1 (E: T) showed significantly higher anticancer cytotoxicity compared to other E: T ratios (5: 1, 2.5: 1 and 1.25: 1), $p<0.0001$. There was also a significant difference in the amount of dead cells (as represented by fluorescence intensity) caused by various concentrations of neutrophils in the study. Isolated TANs showed anticancer cytotoxicity even at the lowest E: T = 1.25:1 ratio.

As previously discussed in the review, neutrophils can kill cancer cells through various mechanisms, including deployment of toxic components such the NADPH oxidase complex, superoxides, H_2O_2 and HOCl [80, 121]. The cytotoxic activity of neutrophils reached a plateau after 2.5 h and started to decrease. Too many dead cells per well (neutrophils + NeuTL cells) may have interfered with the plate reader signal at the later time points. Although neutrophil cytotoxicity at E: T = 20:1 was higher even within the first 30 min, the results were not included because of the increased number of

background dead cells per well (neutrophils+ NeuTL cancer cells) which was caused inaccuracy in the readings (signal interference). In the future improving the process of purification (to > 90) of neutrophils separated from tumour cells may help reduce signal interference on the plate reader.

To determine the specific killing of the cancer cell targets the following formula was used:

$$\% \, Specific = \frac{[(NeuTL\ murine\ cancer\ cells + Neutrophils) - (Neutrophils\ alone) - (100\ \%\ Live\ Cancer\ Cells)\ ]}{(100\ \%\ Dead\ Cancer\ Cells - 100\ \%\ Live\ Cancer\ Cells)} x100$$

The results showed neutrophil specific killing over the E: T ratio range. The % specific killing of neutrophils at 10:1 (E: T) was significantly higher ($p < 0.01$) compared to E: T = 2.5: 1and 1.25:1 and when comparing E: T = 5: 1 vs E: T = 1:1.25 ($p<0.05$). However, there was no significant difference when comparing E: T =10: 1 vs E: T = 5: 1, 5:1 vs E: T = 2.5: 1 and E: T = 2.5:1 vs E: T =1.25: 1 ($p>0.05$).

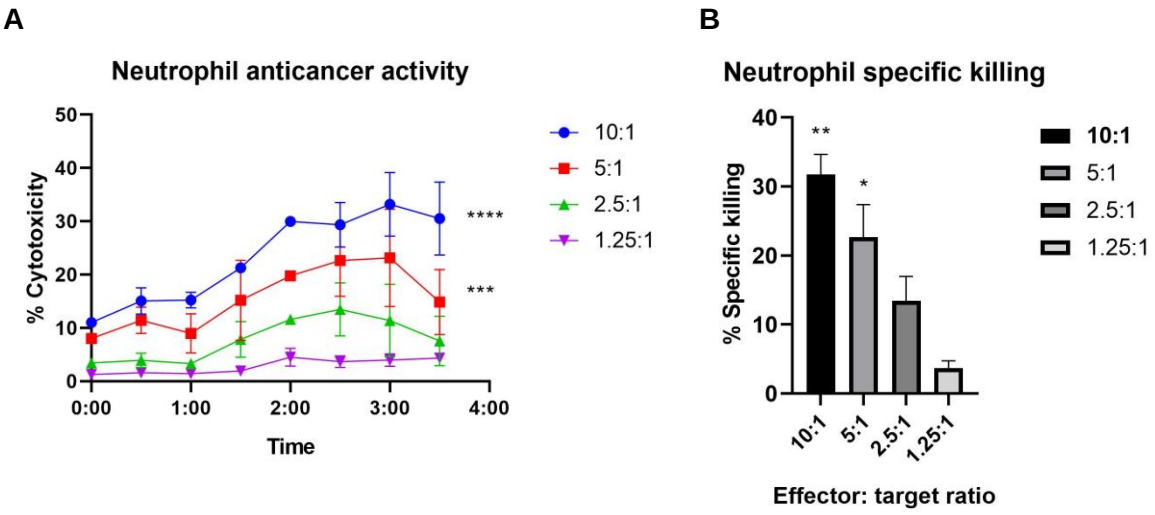


Figure 16: The effect of co-culturing NeuTL murine cancer cells with TANs isolated from treated FVB/N mice c-neu

The cytotoxicity of TANs isolated from treated tumours of FVB/N c-neu mice towards NeuTL murine cancer cells was assessed by measuring the uptake of SYTOX Green nucleic acid stain by dead cells. For analysis, n=3 replicates per assay point were used. Fluorescence Intensity was measured in a plate reader (BMG FLUOstar OPTIMA, Australia) every 30 min for 3.5 h, using the 488 Argon-ion laser with excitation and

emission set at 504/520-10 nm respectively. (A) Fluorescence intensity measurements (representing level of dead cells) at time intervals from 0 to 3.5 h, at different E: T ratios from 10:1 down to 1.25:1(B) Bar graph comparison of the calculated % specific killing of neutrophils at the E: T from 10:1 down to 1.25:1 (n=3) as indicated. GraphPad Prism v 8.2 was used for analyses. Error bars represent ±SEM and **** = p <0.0001, **= p< 0.01, * = p<0.05.

To examine viability of cells until 48 h, images were captured from the 96 well plate with the OLYMPUS IX53 (cellSens Standard software), using the 10 x objective and fluorescence images were captured in the FITC channel. Images of 100 % Live and 100 % Dead NeuTL murine breast cancer cells were used as controls for comparison (Figure 17 below).

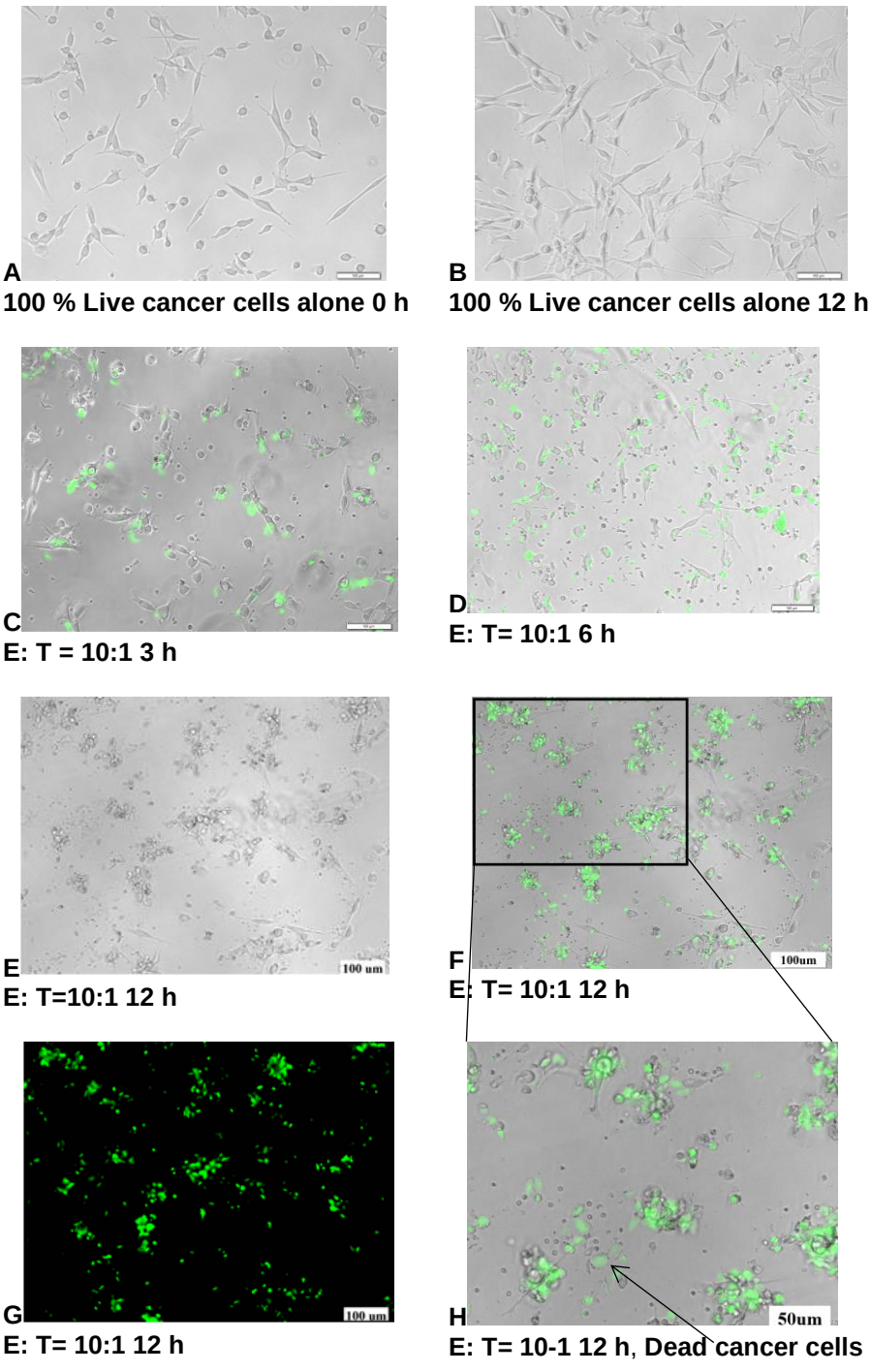


Figure 17: 96 well plate neutrophil cytotoxicity for cancer cells over time intervals until 12 h (A and B) 96 well plate images of 100 % live cells alone at 0 h and 12 h respectively to indicate tumour cells viability. (C and D) E: T = 10:1 well at 3 h and 6 h respectively (E and G) Light microscope and fluorescence images of well 10:1 (Effector: target ratio) at 12 h, (F) Combined live/dead channels and image of E:T = 10: 1 well (H) Zoomed image

(50 µm) of well with E: T = 10:1 shows extensive clumping of neutrophils and cancer cells as well as death of the targeted NeuTL murine breast cancer cells.

From Figure 17, it can be seen that the breast cancer cells cultured alone were very healthy with negligible dead cells in the culture over the time course of the assay (Figure 17 A and C). Traditionally, neutrophils are known to be inherently short-lived (approximately 5–6 days) and undergo spontaneous apoptosis although in infected tissues, their apoptosis can be delayed both by microbial constituents and by pro-inflammatory stimuli [122, 123]. In addition, it would appear that the neutrophils were migrating and clumping together with the targeted breast cancer cells during the course of the assay and were activated to kill the cancer cells, resulting in significant levels of rapidly activated cell death, increasing over the time course of the cytotoxic assay. As discussed in the introductory review section (under the title Neutrophil anticancer cytotoxicity), activated neutrophils have been suggested to form NETs, mobilizing and trapping their targets, to then deploy the toxic mixture of nuclear DNA and granule proteins in high localized concentrations, thereby damaging surrounding cells [89]. The neutrophils also appeared to disrupt as well over the time course of the assay, perhaps indicating the activation of the respiratory burst, leading to the formation of the highly antiseptic HOCL from H_2O_2 by MPO enzymes [124]. Overall, it appears neutrophils were going through different forms of death (apoptosis, necrosis and NETosis), which resulted in the release of toxic concentration of granule and DNA chromatin damaging the surrounding cells over time.

Chapter 5: Discussion and Conclusion

Discussion

The role of the immune system in cancer progression and suppression has been well characterised. However, the role of neutrophils in cancer has been overlooked and overshadowed by research focusing on the adaptive immune cells and antigen-specific cells. Also, most previous studies focused on the influence of tumour associated neutrophils in promoting inflammatory responses and tumorigenesis [67, 68]. Recently TANs have been receiving attention for their role in cancer development and tumour regression. The current study was aiming to expand findings on the potential role of tumour infiltrating neutrophils in mediating tumour regression in transgenic FVB/N spontaneous murine breast cancer models using MAC compound.

As previously stated, the present study was aimed at expanding on previous studies in the Ralph laboratory that investigated the anticancer therapeutic efficacy of MAC using the transgenic FVB/N c-neu spontaneous murine breast cancer model. The study had the following objectives:

1. To determine immunological responses activated by directly injecting MAC into subcutaneous tumours of the female transgenic FVB/N c-neu spontaneous murine breast cancer model.
2. To determine the population of tumour infiltrating neutrophils activated in response to MAC treatment.
3. To assess the nuclear morphological characteristics of activated neutrophils
4. To investigate if activated tumour infiltrating neutrophils have cytotoxic effects against the tumour cells.

In order to complete this study, reliable and convenient methods as well as resources were required. Therefore, a range of protocol optimisation was carried out as detailed in Chapter 3. It was important for the study to obtain enough number of mice with solid tumours

at the same time. Therefore, optimisation of cell injection numbers for establishment of solid tumours was carried out. Furthermore, neutrophils have been described as short-lived in several studies hence, it was important to carry out all studies under sterile conditions to avoid unnecessary activation of neutrophils prior to analyses.

Firstly, the present findings indicated a significant level of tumour growth impairment by the 4 % v/v MAC compound preparation occurred in the treated transgenic FVB/N c-neu spontaneous murine breast cancer models ($p<0.001$). Secondly, the intratumoral injection of FVB/N c-neu mice with 4 % v/v MAC induced an immunological response resulting in an influx of neutrophils into tumours. Thirdly, the tumour infiltrating neutrophils isolated from treated tumours showed a morphological diversity, with hypersegmented neutrophils making up the largest fraction of the isolated cell population. Fourthly, the isolated TANs had a c-Met phenotype consistent with their anticancer activity and showed an extensive and rapid cytotoxicity towards the NeuTL murine breast cancer cells assayed in culture. Together these findings clearly demonstrate the anticancer cytotoxicity caused by MAC and its potential for mediating potent anticancer immune activity.

The antimicrobial and antitumor effects of *Melaleuca alternifolia* essential oil and its derivatives have been reviewed and reported in several studies [12, 15, 16, 103, 109, 111, 112, 117]. For example, *melaleuca alternifolia* and its main bioactive component terpinen-4-ol have been used to inhibit growth of human melanoma, lung, breast, prostate and murine cancer cell lines *in vitro* [11, 12, 26, 111, 112, 117]. Furthermore, intratumoral treatment of subcutaneous non-small lung cancer (NSCLC) cells with terpinen-4-ol inhibited growth of tumours in murine models [111]. Essential oils such as *melaleuca alternifolia* have been shown to modulate multidrug resistance in cancer, which is one of the current limitations with current cancer chemotherapy treatments [103]. For example, terpinen-4-ol inhibited growth of human melanoma M14 WT cells and drug resistant M14 Adriamycin-resistant cells via caspase-dependent apoptosis [12]. Furthermore, topical application of *Melaleuca Alternifolia* as TTO in dilute 10 % dimethyl sulphoxide (DMSO) induced rapid regression of

subcutaneous aggressive AE17 mesothelioma cells in murine models and B16 melanoma cell lines *in vitro* [16, 117].

The current results also indicated the potential selectiveness of MAC compound preparations as demonstrated by the absence of any observed adverse side effects in the experimental mice that received 4 % v/v MAC treatment. Mice from the treatment group did not show any signs of body weight loss due to treatment ($p>0.05$), as the slight decline in overall mice body weights in the treated group was attributed to regression of subcutaneous tumours. The results obtained here are consistent with results from a previous study by a PhD candidate, where MAC was shown to induce apoptosis via the intrinsic mitochondrial pathway and was reported as more selective and potent against human breast and prostate cancer cell lines *in vitro*, compared to terpinen-4-ol after 72 h [14]. In this previous study, MAC treatment was shown to slow down the progression of breast cancers in the treated mice, similar to the results presented here. Nevertheless, the tumours continued to grow.

The population of neutrophils in tumours was also investigated. Tumours harvested from treated mice indicated increased levels of neutrophil infiltration as compared to vehicle control and untreated control groups. Neutrophil infiltration in tumours is very common in most cancers since neutrophils are major players during the inflammatory responses. However, specific events that lead to the influx of neutrophils into tumours as well their specific roles are yet to be fully characterised. In one previous study, topical treatment of aggressive AE17 mesothelioma subcutaneous tumours with *Melaleuca alternifolia* caused tumour regression which was accompanied by a significant influx of neutrophils into tumours and the neutrophils were found to have no impact on tumour regression but were only involved in skin irritation [15]. However, in the previous study of Clark (PhD thesis), the immune mediating effects of 4 % v/v MAC were found to be murine model dependent and was indicated by the influx of neutrophils into spontaneous tumours from the transgenic FVB/N c-neu mice only as well as increased effector T cells in a syngeneic 4T1murine breast cancer model [14]. Furthermore in yet another study, MAC was reported to induce

immune mediated effects by blocking the LPS-induced cytokine production and activation of NF-κB signalling in myeloid derived cell lines *in vitro* [112].

A more recent study indicated that c-Met is required for neutrophil chemoattraction and cytotoxicity in response to its ligand hepatocyte growth factor (HGF) [17]. Hence, c-Met expression on TANs was also investigated by flow cytometry and it was shown here that TANs from all tumours expressed c-Met. However, TANs from tumours treated with 4 % MAC had a higher c-Met expression which correlated with the higher TAN cell counts in the treated tumours.

The current study also assessed the nuclear morphology of isolated neutrophils and the results showed a diverse population of neutrophils, with neutrophils characterised by a hypersegmented nuclear morphology making the bulk of the population (62.72 %). The diversity of circulating neutrophils in cancer has been reported in various studies, which characterised TANs as a heterogeneous population with cytotoxic neutrophils described as having a hypersegemented nuclear morphology [6, 72]. The polarization of neutrophils into tumour promoting and tumour suppressing phenotypes in the absence of TGF-β was first reported by Fridlender *et al.,*2009 and the group went on to describe the phenotypes as N2 (pro-tumorigenic) and N1 (ant-tumorigenic) [6]. Just like TGF-β, a range of cytokine and chemokine factors have been suggested to influence the polarization of neutrophils into antitumor and tumour promoting phenotypes. Type IFN-β was reported to promote differentiation of neutrophils into an anticancer cytotoxic phenotype in patients with melanoma [82]. Furthermore, TNF was also associated with influencing the stimulation of circulating neutrophils from breast cancer patients into cytotoxic phenotypes [79] .

The present study also investigated the functional role of the isolated TANs *ex vivo*. Results indicated the potent anticancer cytotoxicity of neutrophils isolated from tumours of mice treated with 4 % v/v MAC. The neutrophil cytotoxicity was determined at an E: T ranging from 10:1 down to 1.25:1. The isolated TANs showed cytotoxicity at even at the

lowest E: T of 1:25:1, although it was significantly high at 10:1 and 5:1 effector: target ratios ($p<0.0001$ and $p = 0.0299$ respectively). As stated earlier, emerging evidence have been highlighting the potential immune mediating effects of neutrophils in promoting tumour regression. Anticancer cytotoxic neutrophils were indicated to accumulate in the lungs of murine models prior to the arrival of metastatic cells inhibiting seeding of cancerous cells [5]. Furthermore, TANs were suggested to acquire an activated phenotype which enhances T cell activation in patients with early stage lung cancer [70].

Most studies that have explored cancer related neutrophils have largely isolated neutrophils from peripheral blood, while this study was focusing on isolating the neutrophils from drug treated tumours. Isolation of neutrophils from solid tumours using gradient separation proved to be very challenging as a large amount of dead tumour cells and live tumour cells were also found between the interface of Histopaque gradient 1.119 and Histopaque 1.077 g/mL, where greater than 90 % neutrophils are usually isolated from blood samples [74, 75]. Hence, the analysis was hindered by the limitations of the neutrophil purification process.

Cancer immunotherapy goals are centred on the idea of strengthening the immune system of patients with cancers, by improving the ability of immune cells or mediators to recognise and destroy tumour cells. Therefore, it is important to first understand the components and mechanisms of the immune system which are critical for immune mediated tumour rejection.

Concluding remarks

Study limitations

During the present study, dead and live tumour cells were observed co-purifying with the neutrophils, which have an impact on the quality of the neutrophil cytotoxicity assays. Hence, in the future a better method for purification of neutrophils from tumour samples which can achieve > 90 % cell homogeneity should be used. Also, future studies should include investigating molecular mechanisms involved in the cytotoxicity of neutrophils towards cancer cells, such as measuring activity by enzymatic assays for myleoperoxidase levels.

Areas of controversy and limitations

Although studies in mice models have been successful at indicating neutrophil diversity, data on human studies is still limited. The strategy for neutrophil isolation and study comes with its own challenges, including activation of unintended autoimmune responses. Also, with evidence indicating the dual role of neutrophils in cancer, depletion of tumour promoting neutrophils could cause the unintentional down regulation of other critical processes, since the area is yet to be fully understood. Although exploring strategies that focuses on re-education of neutrophils into antitumor cytotoxic cells appears promising, there are still limitations in translating murine tumour models to human pathology.

Areas of agreement and future directions

Although most previous anticancer therapeutic strategies focused mainly on targeting specific T lymphocytes and DCs, the tumour microenvironment and neutrophils as therapeutic targets appears well worth exploring. With accumulating evidence indicating the diversity of neutrophils in cancer with both tumour promotion and antitumor properties, their role in the tumour microenvironment may not actually be as clearly defined as previously suggested. Recent studies have suggested the influence of numerous select cytokines in the modulation of neutrophil function in tumour microenvironments such as tumour necrosis factor (TNF-α), transforming growth factor (TGF)-β and interleukin (IL)-8. Moreover, these

cytokines may also influence neutrophil polarization, although neutrophil function is suggested to be related to the stage of disease. Hence, future studies may need to investigate factors that influence the cytotoxicity of these TANs.

The technique used to purify neutrophils in the present study produced a low yield of neutrophils. In the future optimisation of different techniques aimed at obtaining a purification yield > 90 % is suggested. Furthermore, future studies should aim to explore other possible mediating factors and mechanisms which lead to the killing of cancer cells *ex vivo* by isolated neutrophils. For example, measuring the activity of myeloperoxidase (due to time constraints the present study could not perform this analysis). Future studies could also use neutrophil depletion in mice by injecting the Ly6G 1A8 antibody before treatment with 4 % MAC, to further investigate the role of these immune cells in the tumour micro environment. It will also be interesting to determine if the isolated neutrophils are cytotoxic towards other types of cancer cells.

Conclusion

Overall, the present study was able to indicate the anticancer activity of 4 % v/v MAC *in vivo* using a murine breast cancer model. Injecting 4 % v/v MAC directly into tumours of FVB/n c-neu mice resulted with the infiltration of tumours by $CD11b^{+}Ly6G^{+}$ neutrophils expressing c-Met. The neutrophils had a hypersegmented nuclear morphology and were cytotoxic for murine breast cancer cells *ex vivo*. The isolated neutrophils showed anticancer cytotoxicity and specific killing at E: T and anticancer neutrophil cytotoxicity could be observed at E: T = 1.25: 1. The results showed that neutrophils could be used as a target for immune mediating therapeutic drug development and used in combination therapy to kill residual cancer cells or to reduce burden of tumours before surgery.

Although cancer immunotherapy is proving to be successful in some cancers, it is being faced with limitations including immune related adverse events through nonspecific immunological activation and inflammatory responses. Nevertheless, neutrophils are still

new players in cancer, and hence; may have more unrealized potential for therapeutic targeting of cancers.

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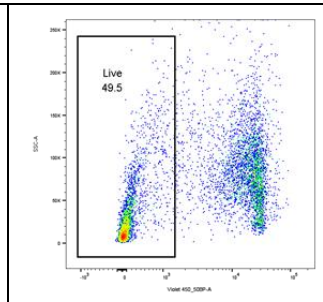
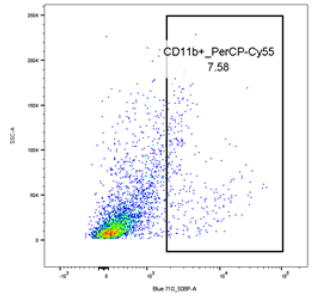
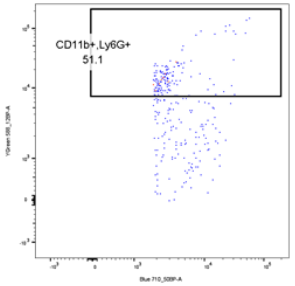
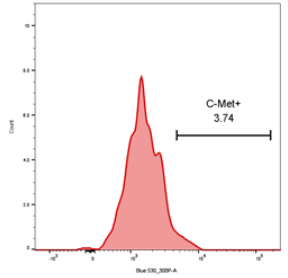
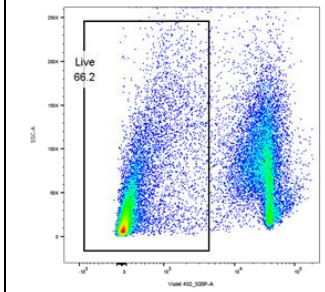
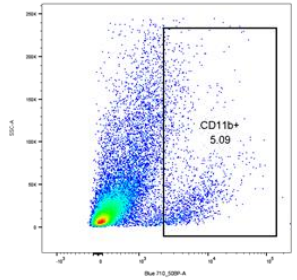
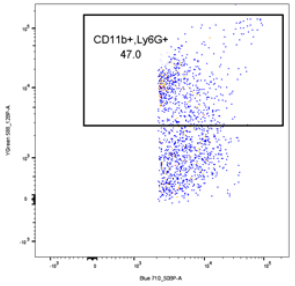
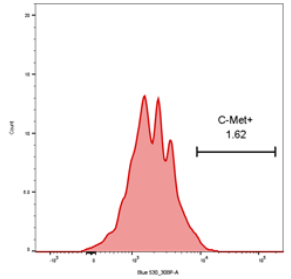
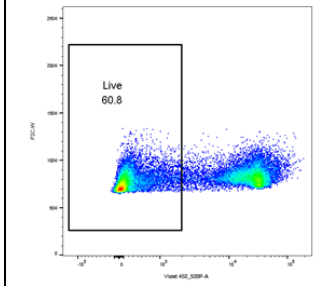
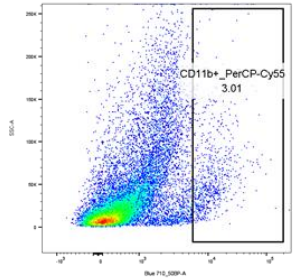
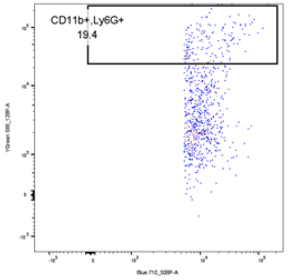
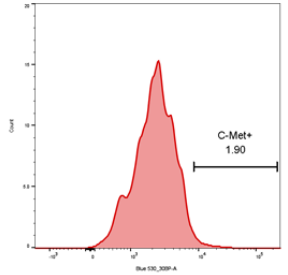
Appendix 1

4 % MAC				
0.5 % TPGS				
Control				
	Live/dead gate	**CD11b$^+$ gate**	**Ly6G$^+$ gate**	**c-Met$^+$ gate**

Flow cytometry identification of TANs from tumours treated with 4 % v/v MAC, 0.5 % TPGS and the control group by

Dot plots represent cell events during analysis. After removal of debris and doublets cells were gated through the live, CD11b$^+$, Ly6G$^+$ and finally the c-Met$^+$ gate.